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Population dynamics, disease and other drivers in a wild snake population

Steven James Ronald Allain

Durrell Institute of Conservation and Ecology
School of Anthropology and Conservation
University of Kent
Canterbury
Kent
CT2 7NR

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Population dynamics, disease and other drivers in a wild snake population

Steven James Ronald Allain

Supervised by
Professor Richard A. Griffiths
Dr Becki Lawson
Dr David I. Leech
Dr David L. Roberts

“I think”

(Darwin, 1837-1838, p. 36)

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Author's declaration

All chapters contained within this thesis were written by Steven J. R. Allain, with editorial suggestions and comments from the supervisory team (Richard A. Griffiths, Becki Lawson, and Dave I. Leech).

COVID-19 impact statement

Just like the rest of the world, COVID-19 had a number of negative impacts and consequences on the progression of my PhD throughout 2020, and beyond. With the onset of the pandemic and the first national lockdown announced on the 23rd March, the following impacts were immediate:

1. Inability to start fieldwork until the 4th July, contracting my field season to after the emergence of snakes post-hibernation.
2. Loss of access to the desktop computer within the Durrell Institute of Conservation and Ecology, that I had been assigned, which I used for analysing large datasets.
3. Loss of access to the laboratories at the Institute of Zoology, Zoological Society of London.

These impacted my ability to (i) collect data upon the emergence of the barred grass snake (*Natrix helvetica*) at the study site, (ii) continue working on the analysis I had started prior to the lockdown (in the end I started from scratch at home), (iii) complete the necessary lab work to inform the 2020/21 field seasons.

The laboratory work described within this thesis was not resumed until October 2021, having been put on hold since the initial lockdown. There was some assistance by one of the internal Institute of Zoology laboratory technicians (a key worker at the Institute) to assist with trouble-shooting while I was unable to travel to London to undertake the work myself (thank Kevin!). Aside from fieldwork preventing access to the laboratories, a global lack of consumables as a consequence of the pandemic, also restricted when the appropriate work could be completed. Due to a lack of time caused by having to squeeze all of my laboratory work into a short space of time, I was also unable to analyse the environmental samples collected, in order to quantify the amount of *Ophidiomyces ophidiicola* DNA present within them.

I had also planned to spend a couple of months at the NERC Biomolecular Analysis Facility (University of Sheffield), having submitted an application in early 2020 to allow for the dietary analysis of the grass snakes, through the use of metabarcoding of prey DNA within faecal samples. This aspect of my project has subsequently been axed, although the samples are still awaiting analysis when time and funds allow.

At the time of the first national lockdown, I was living alone as my international housemates wisely left before the borders closed. This isolation for a period of more than three months had a considerable impact on my mental wellbeing, and subsequent progress with my thesis. This was not helped by the fact that come May, I was paying rent for both a room in Canterbury (where I was marooned), and at another property close to my field site, which I could not use. This stretched my budget to the limit, leading to further disruption.

Despite these challenges, I am happy with the thesis that I have produced within. I know that had these unique and widespread obstacles not presented themselves, that the thesis would look more how I originally envisioned.

Abstract

Anthropogenic activities are currently contributing to global wildlife populations declines in what some scientists have named the sixth mass extinction. Reptiles are not exempt from these declines, with species affected by the combined effects of habitat loss, climate change, over-exploitation, disease, and multiple other factors. In recent decades, emerging infectious diseases have become a more urgent concern on the conservation agenda, especially in those species or populations that are already threatened by human-mediated threats or changes. One example of these is ophidiomycosis, which was first detected in wild snake populations in Great Britain in 2016. The barred grass snake (*Natrix helvetica*) is a widespread species throughout Great Britain that has declined regionally in recent times. This thesis aims to answer: (1) What is the prevalence of skin lesions within the study population and its relationship to ophidiomycosis? (2) How do skin lesions caused by ophidiomycosis vary seasonally in barred grass snakes? (3) How do skin lesions affect the behaviour and survival of snakes? (4) What is the population size and annual survival and detectability of snakes within the population?

A study site comprising an artificial wetland and associated habitats in eastern England measuring approximately 0.5 km^2 in size, was monitored for *N. helvetica* through 264 surveys conducted by five surveyors from 2015 to 2021. A total of 1143 individual snakes were captured and identified by their ventral scale pattern, using Wild-ID. These data were then used to build a capture history to compare annual survival and detectability of snakes with and without skin lesions.

Skin lesions were first identified in the population in 2016, which tested real-time PCR positive for *Ophidiomyces ophidiicola* DNA. Starting in 2019, targeted monitoring of skin lesions was undertaken to investigate their characteristics and aetiology, with snakes showing clinical signs being swabbed for later rtPCR analysis. Skin lesions were observed in 25.5% of individual snakes captured from 2019 to 2021 inclusive. The lesions occurred mainly on the ventral surface of snakes as discoloured areas of mild crusting on scales, or scale margin erosion. When present, a tailored severity classification system was used to score the severity of skin lesions. A total of 78.7% of the swabs taken from snakes with skin lesions, tested rtPCR positive for *O. ophidiicola* DNA. These results were reinforced through the use of histopathology of both tissue and slough samples, which supported use of skin lesions as a proxy for ophidiomycosis in later analyses. Skin lesions occurred more frequently in adults than sub-adults or neonates, and a higher occurrence of trauma was observed in snakes with skin lesions, indicating potential changes in behaviour leading to increased conflict with predators.

Jolly-Seber models indicated a population size that fluctuated between 323-780 (SE: 66.15-179.67) snakes per year. Cormack-Jolly-Seber models showed that snakes with skin lesions had approximately half the apparent annual survival through time ($\varphi = 0.224\text{-}0.225$), than those snakes without skin lesions ($\varphi = 0.531\text{-}0.639$). Moreover, the detectability of snakes with skin lesions was also significantly higher ($p = 0.693\text{-}0.721$) than those without lesions ($p = 0.08\text{-}0.163$). Ophidiomycosis may therefore lead to changes in behaviour that could potentially expose snakes to higher predation pressure.

Finally, the anti-predator behaviour of snakes was compared between snakes with and without skin lesions. Three anti-predator behaviours were observed, with musking being the most frequent, being recorded in over 75% of snakes upon initial capture. There were significant differences between the observed behaviour of snakes between both the capture and processing phases. The response of snakes to initial capture did not vary according to sex, size class, presence of skin lesions, or slough cycle. Snakes were less likely to feign death on subsequent captures than those encountered for the first time. Snakes with skin lesions also feigned death for a longer duration than those snakes without skin lesions.

Changes in the behaviour of snakes affected by ophidiomycosis may lead to reduced survival rates, posing a threat to populations if recruitment is not high enough to compensate. The findings related to the prevalence of ophidiomycosis in *N. helvetica* could also have implications on survey design, such as employing a risk based surveillance strategy to improve the efficiency of ophidiomycosis detection.

Keywords: *Natrix*, snake, species monitoring, ophidiomycosis, survival modelling, apparent annual survival

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Chapter 1. Introduction

1.1 The Anthropocene and the sixth mass extinction

Human impacts on the planet are reflected in the proposed name of the current geological epoch – the Anthropocene (Steffen *et al.*, 2011). This period has been redefined due to the changes observed in the planet's environment since the start of the Industrial Revolution, including global warming, animal extinctions, and alteration in the chemical composition of the world's oceans (Lewis & Maslin, 2015). Other modifications include the release of huge amounts of greenhouse gases, such as carbon dioxide into the atmosphere, that were once previously stored in fossil fuels such as oil and natural gas (Monastersky, 2013).

An alarming indication of the Anthropocene is the dramatic loss of biodiversity (Dirzo *et al.*, 2014). Due to the ongoing declines in wildlife populations, ecosystem functions have been lost or destabilised, leading to a potential sixth mass extinction (Pimm *et al.*, 1995; Ceballos *et al.*, 2015; Payne *et al.*, 2016). This increased rate of extinction has been driven by a number of anthropogenic factors, including an increasing human population and removal of natural resources from the environment. With a reduction in wildlife population size caused by such factors, the genetic diversity of an affected species may also be lowered, making them more susceptible to extinction from secondary stochastic events (Frankham *et al.*, 2002). Other prevailing threats to biodiversity worldwide include pollution, wildlife trade, invasive species, and emerging diseases (McNeely, 1992; Sala *et al.*, 2000; Lenzen *et al.*, 2012; Tilman *et al.*, 2017).

Both marine and terrestrial habitats are impacted by biodiversity loss (Lydeard *et al.*, 1994; Cellabos *et al.*, 2015; Cellabos *et al.*, 2017; Sánchez-Bayo & Wyckhuys, 2019: Antonelli *et al.*, 2020). The IUCN Red List provides a process to assign extinction risk of species at regional and global levels (Gärdenfors *et al.*, 2001; Rodrigues *et al.*, 2006; Schatz, 2009). Biodiversity loss may be addressed through the adoption of multilateral treaties such as the Convention on Biological Diversity (Balmford *et al.*, 2005), whereby signatories agree to implement changes or regional legislation in order to reverse the negative impact their country is having on biodiversity.

Conservation efforts are typically biased towards larger, more charismatic species (Clark & Ray, 2002; McClenachan *et al.*, 2012; Albert *et al.*, 2018), such as birds and mammals, which means that reptiles are often overlooked in this context. Reptiles may be considered less charismatic by some, but they have vital ecological roles within ecosystems, by acting as predators, prey, pollinators and, grazers, (Böhm *et al.*, 2013). The bias seen in reptiles may be due to their small distribution ranges and narrow ecological niches (in general), as well as poor public perception especially where they are considered to be a threat to humans (e.g. venomous species) (Musah *et al.*, 2021). Nearly 15% of reptiles are listed as Data Deficient by the IUCN Red List, indicating that further information is required before their conservation status can be reassessed (Bland & Böhm, 2016; Cox *et al.*, 2022). These tend to be species that are poorly known such as recently discovered or described species, or those with a small population size and therefore a low detectability. It is considered likely that a number of these species are currently threatened with extinction (Howard & Bickford, 2014), despite the paucity of data. It is therefore paramount for the conservation of reptiles to gather more data on population status to inform their conservation assessments.

1.1.1 Reptile taxonomy and conservation status

As of 2022, over 11,000 reptile species were known to science, with more species being described each year (Uetz *et al.*, 2022), and advancing genetic techniques allowing taxonomists to identify cryptic diversity that traditional morphological techniques were unable to identify (Nagy *et al.*, 2012; Oliver *et al.*, 2009). Currently reptiles are split into three different taxonomic orders: Crocodilia (which includes the crocodiles and the alligators), Lepidosauria (which contains the squamates and rhynchocephalians) and finally the Testudines (turtles and tortoises) (Pincheira-Donoso *et al.*, 2013). Of these 10,000 assessed species, 21.1% are currently threatened with extinction, with 14.8% being classified as Data Deficient by the IUCN Red List (Cox *et al.*, 2022). Conservation strategies require information on species distribution and population status, as well as an understanding of life history and habitat requirements (Lips *et al.*, 2003; Andreone *et al.*, 2005). To gain this information, a range of surveys can be undertaken.

1.1.2 Survey methods for reptiles

Reptile surveys can sometimes underestimate the abundance of a species if the biases related to the employed survey methods are not accounted for (Guillera-Arroita *et al.*, 2014). One of the main problems with surveying for reptiles is their low detection probability (Durso & Seigel, 2015). The efficacy of a survey technique will depend on the abundance and behaviour of the species being surveyed, and the habitat it occupies (Hernandez *et al.*, 2006; Crawford *et al.*, 2020). These difficulties in surveying for reptiles has led to lack of comparative and long-term data compared with other taxonomic groups (Gibbons *et al.*, 2000).

1.1.2.1 Artificial Cover Objects

Artificial cover objects (ACOs) placed in favourable habitats can be used to increase the detection of reptiles (Foster, 2012; Willson, 2016). Artificial cover objects can be made from a variety of materials such as plywood or metallic sheets (Foster, 2012), with roofing felt and corrugated steel being most commonly used in the UK (Sewell *et al.*, 2012). Regardless of the material used, the ACOs need to be at least 0.25 m^2, usually being deployed in groups or arrays (Reading, 1997; Foster, 2012; Willson, 2016). Once placed on the ground within a targeted study area, they should be regularly checked for the presence of target species, during their active period (Engelstoft & Ovaska, 2000; Foster, 2012).

Artificial cover objects create a favourable refuge where reptiles may thermoregulate, digest food, and avoid predators (Foster, 2012). Reptiles may use ACOs to behaviourally thermoregulate in several ways. As ectotherms, reptiles may shuttle between basking and seeking refuge (i.e. heliotherms); or may absorb heat directly from an object in the environment (i.e. thigmotherms), allowing them to maintain a preferred body temperature (Brattstrom, 1965, Spellerberg, 1972; Raske *et al.*, 2012). These different physiological and behavioural thermoregulatory strategies dictate how a target species uses an ACO (Reading, 1997).

Artificial cover objects are relatively easy to use with minimal training, low maintenance, and also help to standardise the level of survey effort that can be repeated through time, and aid to reduce observer biases that other methods are prone to (Foster, 2012; Willson, 2016). They are typically employed to monitor a population through both space and time, and can be used in conjunction with other techniques such as mark-recapture in order to estimate the detectability of the target species (Mazerolle *et al.*, 2007).

The disadvantages of using ACOs include the fact that they rely on ambient temperature to warm them, which means they may allow reptiles to very quickly reach their preferred body temperature on a hot day, and be less dependent on ACOs to maintain this temperature (Beauchat & Ellner, 1987; Joppa *et al.*, 2009). A number of other climatic factors such as rainfall, cloud cover, and wind speed may also affect the ability of ACOs to attract reptiles (Joppa *et al.*, 2009). Artificial cover objects may also be lost in areas where vegetation grows quickly, or due to flooding of a site. Unfortunately ACOs may also be tampered with by members of the public, disrupting surveys and potentially the target species (Willson, 2016).

1.1.2.2 Visual encounter surveys

A number of reptile species avoid ACOs, meaning that alternate methods are needed in order to survey for them, such as visual encounter surveys (VES; Doan, 2003). These can vary in their approach depending on the habitat and target species, but usually involve walking along a given transect at a set pace (in both space and time), whilst carefully observing the habitat for reptiles (Doan, 2003; Willson, 2016). Species are often seen basking on the ground, in bushes or other vegetation features which are present in the environment. Visual encounter surveys also have their limitations, e.g. if the target species is arboreal then there is a risk of a missed detection. Surveyors need to be trained in the quick identification of species, the natural history of target species, and activity patterns, as reptiles are likely to react to disturbance and seek refuge (Willson, 2016).

1.1.2.3 Pitfall Traps

Pitfall traps are usually buckets dug into the ground with a drift-fence that guides reptiles to the pitfall (Enge, 2001). This works well for smaller species (Dundas *et al*., 2019), but larger ones may easily be able to escape (depending on how large the pitfalls are) or they may eat other species within the pitfall trap leading to reduced counts (Enge, 2001; Dundas *et al*., 2019). Like ACOs and VES, the weather can influence the number of reptiles detected – mainly due to their physiology, with temperate reptiles being less active in colder or wetter conditions (Bury & Corn, 1987). Pitfall traps often perform well when they are combined with other methods for detecting and collecting reptiles for examination before release (Raxworthy & Nussbaum, 1994).

1.1.2.4 Radio-tracking

Radio-tracking is a method that has been used to monitor the movement of reptiles over varying periods of time with the transmitters implanted internally, or attached externally (Doody *et al*., 2009). A Home Office licence is required for the surgical implantation of radio-transmitters within snakes in the UK. Due to both ethical and practical reasons, radio-transmitters are sometimes attached externally to terrestrial snakes (Tozetti & Martins, 2007), with the practice of attachment favoured within the UK. Attachment methods for external transmitters include the use of substances such as glue, thread, tape and other such materials (Riley *et al*., 2017). Radio-tracking can be used to determine the home-range of a species, habitat use and the location of egg laying or hibernation sites (Madsen, 1984). This is particularly useful for investigating spatial ecology of a species (Gent & Spellerberg, 1993). Internal implantation of transmitters have been shown to not cause any significant changes in the behaviour or movement of tagged animals (Lutterschmidt, 1994), and may be more appropriate for aquatic species.

The limitations to the use of radio-transmitters include their transmitting distance and the life of the batteries installed in each unit. Although battery technology has advanced in recent decades, transmitters are still limited by the size and life of the batteries they are fitted with (O'Mara *et al*., 2014). As they tend to be attached externally, they may detach in dense habitat or when the reptile sloughs (Tozetti & Martins, 2007; Wylie *et al*., 2011).

1.1.2.5 Other survey methods

There are a number of other less frequently used survey methods depending on the target species. Most of the methods described above are best suited to terrestrial reptiles, with few applicable for the study of semi-aquatic or aquatic reptiles. Aquatic species are harder to survey, due to their inaccessibility but new techniques such as environmental DNA (eDNA) may help to reveal species distribution by providing presence/absence data (Davy *et al*., 2015). Other technological advancements such as drones can also be employed in the survey of aquatic reptiles that would otherwise be disturbed by the presence of researchers (Bevan *et al*., 2018). Also challenging are arboreal species, which may live high up in the canopy out of sight of researchers. The use of minnow traps placed in trees and spotlighting are methods that researchers have used to combat this challenge (Ribeiro-Júnior *et al*., 2006; Bell, 2009). Alternatively it may be effective to use arboreal cover boards as a means of surveying for species that hide in cracks and crevices (Nordberg & Schwartzkopf, 2015). Since the 1930s, researchers in North America have employed 'road cruising', whereby paved roads are driven along slowly at night, in order to detect reptiles absorbing heat from the road surface (Dodd *et al*., 1989).

1.2 Global causes for reptile declines

1.2.1 Climate change

Climate change is an ongoing threat to most groups of animals and plants, causing the disruption of weather systems, and exacerbation of factors such as habitat loss and introduced species (Bellard *et al*., 2012; Garcia *et al*., 2014). Disruption to ecosystems caused by changes in rainfall, increased temperature and ultraviolet radiation, may all have drastic consequences on reptile populations by the end of the century (Bickford *et al*., 2010). The increased temperatures caused by climate change may lead to reptile declines, with their physiology rendering them unable to adapt to warmer temperatures. This may lead to geographically isolated species (or populations) on mountain ranges suffering from upslope displacement (Raxworthy *et al*., 2008). Reptiles have shown some phenological plasticity in response to climate change, but there are still a number of unknown responses due to a lack of data (Urban *et al*., 2014). In Europe, reptile assemblages have been predicted to move northwards as the continent warms from the south (Araújo *et al*., 2006), with observations of these range shifts already occurring (Moreno-Rueda *et al*., 2012). There is, however, evidence to suggest that some species may benefit from climate change, by expanding their potential distribution ranges (Araújo *et al*., 2006; Ceia-Hasse *et al*., 2014).

Research has already demonstrated that lowland reptile species in the tropics are experiencing the effects of climate change, such as temperatures above their optimum, which may make them less able to compete for resources (Huey *et al*., 2009). In some instances genetic adaptation may be possible to help those affected species counteract the effects of climate change, demonstrating at least that some reptiles are resilient (Urban *et al*., 2014). Examples of detrimental effects due to climate change from Europe, include the documented decline of aspic vipers (*V. aspis*) from a well-known hibernation site in Italy (Luiselli *et al*., 2018), as well as changes to their phenology (Rugiero *et al*., 2013). Collectively, these observations indicate that not all reptiles are affected by climate change in the same way, and that effects are global. The limits of reptiles to mitigate the effects of climate change behaviourally, genetically, or physiologically deserves further research.

These risks are not exclusive to terrestrial species. Marine turtles that come ashore to nest also face extinction risks due to climate change (Fuentes *et al*., 2011). In addition they nest on low-lying beaches that may be flooded due to rising sea levels, and the determination of sex ratios in turtle embryos also makes them vulnerable. Turtles use temperature-dependent sex determination, relying on the nest temperature to regulate their hatching sex ratio, a warming planet has the effect of causing population-wide feminisation (Jensen *et al*., 2018).

Climate change is currently having a mixed effect on wild reptile populations, with most of these being negative, with shifts in home ranges, phenology, and sex ratios among some of the documented impacts. While climate change is a growing threat to reptiles, the most immediate factor contributing to population declines and species extinctions, is habitat loss. Incubation temperature can also affect other reptiles such as snakes, with increased temperatures leading to embryo death, or morphological deformities in hatchlings (Burger *et al*., 1987; Idrisova & Khairutdinov, 2018).

Climate change may also impact the physiological processes of reptiles, with water deprivation caused by weather extremes such as drought, linked to reduced embryonic development and mortality in northern adder (*Vipera berus*) in experimental settings (Dezetter *et al*., 2021). Using simulated winter conditions under different scenarios to mimic the impacts of climate change, Brischoux *et al*. (2016) found that mild wintering conditions (at approximately 14°C) induced a marked decrease in body condition, and provoked an alteration of some hormonal mechanisms involved in emergence compared to cold winter conditions (approximately 6°C), over the period of a month in the aspic viper (*V. aspis*). Finally, the combined effects of droughts and heatwaves were simulated in *V. aspis*, demonstrating that a short-term exposure to combined heatwave and drought can exacerbate physiological stress through additive effects (Dezetter *et al*., 2022). Such effects are likely to bear ultimate consequences on survival and reproduction, and thus population persistence.

1.2.2 Habitat loss

Many reptile species are susceptible to a reduction in the availability or fragmentation of habitat, due to their thermal requirements, low dispersal ability, and limited distribution (Driscoll, 2004; Kearney *et al*., 2009; Maritz *et al*., 2016; Paterson *et al*., 2021). Habitat loss also leads to a diminished amount of available prey, which can cause declines in species that are in higher trophic positions (Reading & Jofré, 2020). Fragmentation of habitat may also lead to a decline in gene flow between populations (Smith *et al*., 2009). However, some species are able to persist in matrices of degraded habitat, although their success is linked to the quality of these fragments (Lion *et al*., 2016).

Large-scale deforestation is a global issue, which often takes place to expand the amount of land being used by pastoral farming, and to provide land for the growing of crops such as soya and palm oil (Vijay *et al*., 2016). With the loss of primary or secondary rainforest comes the reduction in suitable habitat for reptile species with small-ranging species the most vulnerable to extinction (Brooks *et al*., 2002). Some well-documented examples are the deforestation of Madagascar with 40% of the forest cover being lost in the latter half of the 20th Century (Harper *et al*., 2007), and Singapore which has seen the extinction of a number of endemic reptiles due to a 95% loss in original forest cover (Brook *et al*., 2003).

1.2.3 Introduced species

The establishment of an introduced species can have negative impacts on the native fauna (Gibbons *et al*., 2000). For some species, this may be due to the introduced species being a novel predator, as is the case with non-native rats (*Rattus norvegicus* and *R. rattus*), feral cat (*Felis catus*), and Indian mongoose (*Herpestes auropunctatus*) which have extirpated numerous reptile species on islands, and mainland continents (Case & Bolger 1991; Barratt, 1997). The introduced species may also compete for resources with native species if they fill a similar ecological niche, such as food, shelter, or nesting sites (Norbury, 2001; Smith *et al*., 2012). A reduction in the availability of these resources will likely lead to a decline in the native species' reproductive rate. The potential for introduced species to adversely impact global reptile populations is significant, based on current evidence (Kraus, 2015).

Conversely, reptiles have the ecological ability to act as an invasive species when moved outside of their native range, such is the case of Burmese pythons (*Python bivittatus*) in Florida, and brown tree snakes (*Boiga irregularis*) in Guam (Dorcas *et al*., 2012; Wiles *et al*., 2003). When a species undergoes predator release coinciding with the introduction to a new area, that species can potentially cause ecological disruption (Ricciardi & Cohen, 2007). The environment that a species is introduced into has to mimic that of its native range otherwise viable populations may not establish (Reed, 2005). Introduced gecko species (*Hemidactylus frenatus* and *Lepidodactylus lugubris*) are outcompeting local, native species on certain Pacific islands (Case & Bolger, 1991). Those species and populations that occur on islands are particularly at risk of extinction due to introduced predators and can only recover once the threats have been removed (Reardon *et al*., 2012).

1.2.4 Over-collection and the pet trade

Many reptile species are very attractive and there is a growing worldwide market for captive housing and breeding of a number of species (Tapley *et al*., 2011). One way to meet these demands is to breed species in captivity for sale within the pet trade, however, this strategy is not applicable for species for which their husbandry or breeding cycles are currently unknown. Consequently, a high proportion of animals that supply the European and global demand for rare and exotic species are wild caught individuals, with over 7 million wild-caught reptiles imported into the US between 1998 and 2002 alone (Schlaepfer *et al*., 2005;

Auliya *et al*., 2016). Most global exports of reptiles come from less-industrialised countries in the tropics such as Indonesia, El Salvador and Colombia that feed the demand not only in Europe but also China, and the USA (Robinson *et al*., 2015).

Over-collection for the pet-trade is a serious problem facing some species, leading to drastic declines of wild populations. This is especially true to those reptiles that are rare, colourful, or desirable (Auliya *et al*., 2016). One dramatic example of this is the ploughshare tortoise (*Astrochelys yniphora*), which has seen illegal confiscations increase as the wild population has significantly decreased (Mandimbihasina *et al*., 2018). In order to combat the growing trend of poachers using the scientific literature to identify where new and profitable species have been found, some authors have started to omit type locality information from species description publications (Yang & Chan, 2015).

Between the years 2004–2014, over 20 million live reptiles were imported to the EU, with the majority of these entering Germany (Auliya *et al*., 2016). The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) is an international treaty limiting the trade in wildlife, with the aim of protecting many traded species including reptiles and other wildlife from exploitation. In a small number of cases, the listing process and lack of cooperation of CITES parties can lead to an increased risk of extinction in desirable species, if trade quotas are not initiated quickly enough (Janssen & Krishnasamy, 2018).

The global trade in reptiles worldwide has mediated their escape into the wild in new environments, where they may spread non-native diseases or parasites or act as an invasive species (Corn *et al*., 2011). A dramatic example of this is Florida in the United States where 51 species of introduced reptiles (four turtles, 1 crocodilian, 43 lizards, and three snakes) are now established, although many more introductions have failed to lead to self-sustaining populations (Krysko *et al*., 2011), which are impacting local ecosystems. This includes species such as the Nile monitor (*Varanus niloticus*), green iguana (*Iguana iguana*), black spiny-tailed iguana (*Ctenosaura similis*), and northern curlytail lizard (*Leiocephalus carinatus armouri*), among many others (Engeman *et al*., 2011).

Invasive reptile species in Florida have also introduced the pentastome *Raillietiella orientalis*, which is a non-native parasite to the region (Miller *et al*., 2018). The current evidence suggests that spillover occured from *P. bivittatus* to native snakes, which are now being impacted through both direct and indirect consequences of this parasite interaction

(Miller *et al*., 2018). *Raillietiella orientalis* may also be subject to spread within the United States through the trade in wild-caught reptiles, from those areas where *R. orientalis* has been introduced (Farrell *et al*., 2023).

The collection of reptiles from the wild is mainly a problem for countries in the tropics, which boast a higher diversity of species, some of which are colourful and attractive (Robinson *et al*., 2015). This is less of a problem in Europe, yet reptiles are still in decline across this continent, due to a combination of the other factors described above.

1.2.5 Combined threats

There is a strong likelihood for synergistic effects of the threats described above on reptile populations, given the evidence provided by amphibians. Environmental temperature is known to significantly affect disease resistance in reptiles, with increasing temperatures likely to impact pathogen resistance and emergence (Bickford *et al*., 2010). *Ranavirus* is a virus that affects ectotherms such as amphibians and reptiles, although amphibians are most negatively impacted (Price *et al*., 2017). As climate change advances, it is likely that ranavirosis outbreaks will occur over wider areas and for an extended season, within the United Kingdom (Price *et al*., 2019). While these outbreaks are unlikely to cause direct mortality in those reptile species that prey on amphibians, the impacts may be indirect, due to a reduced prey availability within the environment, impacting predator survival and fitness.

In some areas of the world such as Southeast Asia, which has a high level of reptile endemism, small populations which are threatened by the combined effects of habitat loss, increasing temperatures, and decreased precipitation, could lead to localised extinctions (Bickford *et al*., 2010). These interactions between different stressors indicate how they can be amplified via additive effects, to impact vulnerable populations. In some cases it is hard to disentangle which threat is the main driver of decline, so that it can be mitigated effectively.

1.3 Reptile declines within Europe

Reptiles are found in every country within Europe (except Iceland) and populate a majority of the islands within this geographical region. Reptile species can be found throughout the continent including within the Arctic Circle, such as the *V. berus* and the viviparous lizard (*Zootoca vivpara*), with the highest diversity of species being found in the Balkans (Džukić & Kalezić, 2004). Of the approximately 150 reptile species that occur in Europe, 27 (18%) are currently threatened with extinction (Cox & Temple, 2009). The recent taxonomic split of the grass snake into two distinct species (*Natrix natrix* and *Natrix helvetica*) and other such taxonomic revisions may mean that the actual figure may be slightly higher, as the current Red List assessments need updating to account for these changes.

Whilst most reptile species are widespread in Europe, they are declining throughout their range due to threats such as agricultural intensification, increasing urbanisation, and habitat loss (Falaschi *et al*., 2019). Other less studied and potential causes for decline include environmental pollution, such as that from pesticides (Wagner *et al*., 2015; Mingo *et al*., 2016), and heavy metal poisoning (Lettoff *et al*., 2021). Some snake populations (including both colubrids and viperids) have been found to be in decline within Europe, but the immediate cause for these declines is not yet known (Reading *et al*., 2010). Grass snake (*N. natrix*) declines in Sweden have been linked with changes in agriculture, with the intensification of farm management from small-holdings to much larger enterprises, and the loss of potential nesting habitat for snakes that they provide (Hagman *et al*., 2012).

1.3.1 Reptile conservation in the United Kingdom

There are six native reptile species in the UK (Beebee & Griffiths, 2000), comprising three snake species (smooth snake; *Coronella austriaca*, barred grass snake; *Natrix helvetica* and northern adder; *Vipera berus*) and three lizard species (slow-worm *Anguis fragilis*; sand lizard *Lacerta agilis*; and viviparous lizard *Zootoca vivipara*). *Anguis fragilis*, *N. helvetica*, *V. berus*, and *Z. vivipara* are widely distributed, whereas *C. austriaca* and *L. agilis* are restricted in their range due to being habitat specialists (Spellerberg, 1975). Declines in widespread reptiles in the UK are mainly due to the effects of habitat loss and fragmentation, caused by the development of land for housing and infrastructure (Foster *et al*., 2021). The smooth snake (*C. austriaca*) and sand lizard (*L. agilis*) are the two most range restricted reptiles in the UK, being found mainly on heathland sites in the south of the country, with *L. agilis* also being found on dune systems in northern Wales and north-west England (Beebee & Griffiths, 2000). In recent years, efforts to reintroduce *L. agilis* have been successful at 21 sites across southern England (Woodfine *et al*., 2017).

The decline of adders (*V. berus*) in the UK has been recognised since the 1980s (Cooke & Arnold, 1982), but recent evidence suggests that small populations are far more vulnerable than larger ones (Gardner *et al*., 2019). The current population trends for the barred grass snake (*N. helvetica*), viviparous lizard (*Z. vivipara*) and slow-worm (*A. fragilis*) are not currently known, however it is believed that like the adder these three species are currently experiencing localised declines (Beebee & Ratcliffe, 2018). All three are currently listed as Least Concern on the UK Regional Red List (Foster *et al*., 2021). There is however a growing need for the implementation of landscape-scale reptile conservation efforts, as so far most efforts in the UK have been quite localised. The largest project so far assisting in informing conservation interventions of reptiles is the ongoing monitoring of adder populations in Great Britain entitled ‘Make the Adder Count’ (Gardner *et al*., 2019).

Additionally, all six native reptile species are currently listed under Schedule 5 of the 1981 Wildlife and Countryside Act, which provides legal protection from sale, harm and intentional disturbance (HM Government, 2022). Habitat management can be used as a beneficial strategy to help improve areas where reptiles occur, although not all management practices will benefit every species equally (Jofré *et al*., 2016).

1.3.2 The barred grass snake (*Natrix helvetica*)

Great Britain’s largest native terrestrial reptile, the barred grass snake (*N. helvetica*), known locally as the grass snake, is widespread throughout England and Wales with a limited distribution in Scotland (Beebee & Griffiths, 2000). The remainder of their distribution includes much of Western Europe, including France, Belgium, the Netherlands, Switzerland, Italy, and western Germany (Kindler *et al*., 2017). Grass snakes can grow to over a metre in length, but are more typically 70-80 cm long with females being larger than males (Beebee & Griffiths, 2000). Generally, they are olive, green or brown in colour with two rows of dark markings along the flanks of the body, a yellow, orange, or white collar at the back of the head, followed by a pair of black crescents (Boulenger, 1913; Steward, 1971; Beebee & Griffiths, 2000). The ventral scales of grass snakes are white or a pale cream colour, with irregular dark markings, which can be used to help differentiate individuals (Carlström & Edelstam, 1946).

The preferred habitat of grass snakes consists of wetlands, open deciduous woodlands, heathland and rough grassland (Beebee & Griffiths, 2000), with the species being closely associated with water, due to a diet primarily consisting of amphibians (Reading & Davies, 1996). Grass snakes also predate a range of other prey; for instance fish, small mammals, birds, gastropods, and even other reptiles (Gregory & Isaac, 2004; Consul *et al*., 2009). The composition of their diet changes in relation to ontogeny, and the time of year depending on prey availability (Gregory & Isaac, 2004). Grass snakes are active from March until October, with courtship and mating occurring through to May, eggs being laid in decomposing vegetation such as compost heaps between June and August, and hatchlings emerging in late August to September (Steward, 1971; Beebee & Griffiths, 2000).

Grass snakes are the most frequently encountered snake in England, which may help to explain why they are generally regarded as being quite common. There are concerns of localised declines in some parts of the country, especially in northern England (Durkin, 2016; Foster *et al*., 2021). The current threats identified as affecting grass snake populations include the loss and fragmentation of habitats, deterioration of egg laying sites, and the presence of non-native predators such as domestic cats (Foster *et al*., 2021). Positive conservation interventions for grass snakes therefore involve improving the quantity and quality of open habitat and wetlands, and increasing the provision of egg laying sites within those environments (Foster *et al*., 2021).

1.4 The emergence of novel diseases in modern times

Emerging infectious diseases (EIDs) are another factor contributing to the decline of wildlife populations, including reptiles (Daszak *et al*., 2000; Gibbons *et al*., 2000; Fisher & Garner, 2020). The majority of EIDs that are of conservation concern are caused by fungal pathogens (Fisher *et al*., 2012), although viral and bacterial emerging pathogens have also been identified. Reptiles are not alone in being subjected to recently emerged pathogenic fungi: amphibians (Fisher & Garner, 2007; Martel *et al*., 2013), and bats (Hoyt *et al*., 2021) have also been affected within the past three decades. The occurrence of EIDs has been linked to factors such as climate change (Epstein, 2001; Pounds *et al*., 2006), pollution (Anderson *et al*., 2004), and global trade in wildlife products (Daszak *et al.*, 2001; Karesh *et al*., 2005).

1.4.1 Emerging Infectious Diseases in amphibians

The amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) is widely considered the most ecologically damaging EID in terms of impact on vertebrate species biodiversity that has been identified in the past 30 years. This amphibian chytrid fungus was first identified in 1998 (Berger *et al*., 1998) and scientifically named the following year (Longcore *et al*., 1999). Since then, *B. dendrobatidis* has been implicated in the declines of thousands of species worldwide and the extinction of approximately two hundred amphibian species (Wake & Vredenburg, 2008), although this is debated and may be closer to 90 (Scheele *et al*, 2019; Lambert *et al*., 2020). There are a number of endemic strains of *B. dendrobatidis* (in Brazil, East Asia, and South Africa), but the strain that has been mainly linked to global amphibian declines has been named the Global Panzootic Lineage (*Bd*GPL) (Fisher *et al*., 2009b). The fungus infects keratinised skin, a vital organ for amphibians that also leads to metabolic disruption and finally death (Piotrowski *et al*., 2004; Grogan *et al*., 2018). It has long been debated where *Bd*GPL originated but recent research indicates that the most likely point of origin is the Korean Peninsula (O'Hanlon *et al*., 2018). Unfortunately the disease has been spread unwittingly around the world due to the trade in amphibians for uses such as food or the pet trade (Fisher & Garner, 2007). Research has shown that the fungus now has a global distribution (Fisher *et al*., 2009a) and a wide host range (Olson *et al*., 2013).

The amphibian chytrid fungus is not the only major fungal pathogen to infect amphibians, with the recently emerged salamander chytrid fungus (*Batrachochytrium salamandrivorans*) first identified in 2013. After a dramatic decline in European fire salamanders (*Salamandra salamandra*) in the Netherlands, the pathogen was identified as the cause of mass mortality of this species (Martel *et al*. 2013). Like its sister species, *B. salamandrivorans* is believed to have been introduced to Europe via the pet trade before spillover into wild salamander populations (Martel *et al*., 2014), with *B. salamandrivorans* being the predominant chytrid fungus infecting Vietnamese salamanders (Laking *et al*., 2017). The experimental infection of Asian newts (*Cynops cyanurus*, *C. pyrrhogaster* and *Paramesotriton deloustali*) identified that these species were potential reservoirs of *B. salamandrivorans* (Martel *et al*., 2014).During the same experiments most European newt species died shortly after infection with *B. salamandrivorans* zoospores (Martel *et al*. 2014).

The clinical signs of *B. salamandrivorans* are varied but include anorexia, lethargy, ataxia and visible skin lesions which present as multifocal epidermal erosions or ulcerations (Martel *et al*. 2013). *Batrachochytrium salamandrivorans* has been shown to have a poor dispersal potential which can allow sub-populations to persist in areas where there is a known high prevalence of the infection (Spitzen-van der Sluijs *et al*., 2018), with the fungal spores being environmentally resistant (Stegen *et al*., 2017). Currently *B. salamandrivorans* is only known to be in the wild in Belgium, Germany and the Netherlands although research indicates that it is more widespread in captive collections (Fitzpatrick *et al*., 2018), with the potential for spillover into wild populations.

1.4.2 Emerging Infectious Disease in reptiles

Terrestrial reptiles are an understudied group regarding our knowledge of infectious diseases that may impact wild populations. To date, the majority of studies of diseases in reptiles have focussed on captive animals, due to the relative ease of studying them compared to their wild counterparts. However, observations of morbidity and mortality in the field, and development of wildlife disease surveillance schemes incorporating reptiles, working with herpetologists and ecological consultants, has started to fill this knowledge gap globally in recent years. With an increased knowledge of potential pathogens affecting wildlife, and more sensitive methods of detection (such as qPCR and eDNA), contemporary research has identified a number of novel agents in both wild and captive reptiles (Cabañes *et al*., 2014).

Examples of these novel emerging pathogens include viral agents such as the nidovirus Bellinger River virus isolated from freshwater snapping turtles (*Myuchelys georgesi*) in New South Wales, Australia (Zhang *et al*., 2018), and Serpentovirus in captive snakes across three snake families (Boidae, Colubridae, and Pythonidae) in collections in the USA (Hoon-Hanks *et al*., 2019). Bacterial pathogens include *Chlamydia* spp. (Inchuai *et al*., 2021) and *Salmonella* spp. (Briones *et al*., 2004), both of which may also have implications for human-health. Finally, fungal pathogens affecting reptiles include *Emydomyces testavorans* identified from freshwater turtles in North America (Woodburn *et al*., 2019), and *Paranannizziopsis australasiensis* in captive African bush vipers (*Atheris squamigera*) in the USA (Díaz-Delgado *et al*., 2020).

The occurrence of diseases caused by these emerging pathogens is likely to be exacerbated by factors such as climate change and habitat modification (Lips *et al*., 2008), and pathogens may be spread around the globe through the trade in live reptiles or their derivatives (Fisher & Garner, 2007; Rodgers *et al*., 2011), if amphibians act as an accurate predictor of the future.

1.4.2.1 Ophidiomycosis

Ophidiomycosis (commonly known as snake fungal disease or SFD) is the best known, and most important fungal pathogen that infects reptiles (Paré & Sigler, 2016). *Chrysosporium ophiodiicola* was first identified in 2009, and subsequently reclassified in its own genus and renamed *Ophidiomyces ophidiicola* (*Oo*) in 2013 (Rajeev *et al*., 2009; Sigler *et al*., 2013). This fungus was confirmed as the causative agent of ophidiomycosis through fulfilment of Koch's postulates (Lorch *et al*., 2015). Koch's postulates are four criteria designed to assess whether a microorganism causes a disease within another organism. First, the microorganism must only occur in every case of the disease and not be found in healthy individuals; second, the microorganism can be isolated from a diseased organism; third, inoculation in a healthy individual recapitulates the disease; and finally, the proposed pathogen can be reisolated from this inoculated individual (Byrd & Segre, 2016). The current evidence suggests that *Oo* only has the ability to infect and cause ophidiomycosis in snakes, although further research is needed to confirm the range of susceptible species (Burbrink *et al.*, 2017).

There are a number of clinical signs associated with ophidiomycosis that may vary between species or populations, such as skin lesions and subcutaneous nodules (Lorch *et al.*, 2015). Once *Oo* has penetrated the epidermis (challenge studies indicate that mechanical abrasion to the *stratum corneum* may facilitate infection), the host's immune response then leads to the development of skin lesions (Lorch *et al.*, 2015). Snakes may increase their sloughing frequency as a response to the development of ophidiomycosis in an attempt to shed the pathogen and clear their infection, which has an increased energetic cost (Lorch *et al.*, 2015). There is also evidence to suggest that infection with ophidiomycosis may alter the behaviour of snakes, making them more likely to come into conflict with potential predators, potentially leading to indirect mortality (Tetzlaff *et al.*, 2017).

The appearance of skin lesions is not pathognomonic: combined histological and genetic evidence is required to confirm a diagnosis of ophidiomycosis, which is typically completed through the post-mortem examination of wild snakes (Allender *et al.*, 2011). Snake skin sloughs offer an alternate way to detect *Oo* DNA via real-time PCR (Dibadj *et al.*, 2021).

The USA was the first country where ophidiomycosis was diagnosed in wild snakes (Dolinski *et al.*, 2014; Rajeev *et al.*, 2009), with the disease originally restricted to the eastern States. Now however, it seems that ophidiomycosis may have a wide distribution after a confirmed diagnosis in California (Haynes *et al.*, 2021). Ophidiomycosis may also be linked to declines in both populations and genetic diversity within smaller populations of more susceptible species (Clark *et al.*, 2012). Recent retrospective analysis of museum specimens in the USA has revealed that confirmed cases of ophidiomycosis in wild snakes date back to at least 1945 (Lorch *et al.*, 2021). Genetic evidence supports multiple recent introductions of *Oo* to North America, which may be linked to the trade in captive reptiles (Ladner *et al.*, 2022). Elsewhere in the world, ophidiomycosis has been confirmed in captive and wild snakes in Asia (Grioni *et al.*, 2021; Ovchinnikov *et al.*, 2021; Takami *et al.*, 2021), Australia (Sigler *et al.*, 2013), and Canada (Davy *et al.*, 2021).

Due to the growing concerns of conservation biologists, disease surveillance of wild snakes in Europe has recently resulted in the diagnosis of ophidiomycosis in a small number of species (Franklinos *et al*., 2017; Meier *et al*., 2018). Currently, ophidiomycosis has been identified in three *Natrix* species (*Natrix helvetica*, *N. maura*, and *N. natrix*) in three countries (Czech Republic, Switzerland and the United Kingdom). It is not known how widespread ophidiomycosis is in Europe, whether *Oo* is an endemic or introduced pathogen, what the impact on both host health and population status is, and whether environmental stressors (e.g. climate change) may influence disease occurrence (Lorch *et al*., 2016; Ladner *et al*., 2022). So far, the knowledge of ophidiomycosis in Europe has been limited to post-mortem examinations with a lack of live snake surveillance, and therefore, more evidence to inform its impacts are needed through the use of longitudinal field studies.

1.5 Aims and objectives

In order to assess population dynamics of barred grass snakes (*Natrix helvetica*) and the potential drivers (such as disease), a site in Eastern England was chosen. The research site has a large population of *N. helvetica* that has been studied since 2015, with *Oo* first confirmed from skin swabs taken from infected snakes in 2016. Through surveys conducted for *N. helvetica* and historical data, this thesis intends to answer the following questions:

- What are the characteristics of skin lesions in captured snakes, and what is the prevalence of ophidiomycosis in the population?
- What are the drivers of survival and detectability in the population, and how are these affected by the presence of skin lesions?
- What are the behavioural responses to capture observed by barred grass snakes, and is this influenced by ophidiomycosis?

Chapter 2. Survey protocols and population structure in the barred grass snake (*Natrix helvetica*) at a site in Norfolk, England

2.1 Abstract

A challenge in analysing long-term population data is determining the consistency in observations made by different observers over different survey windows. For a seven-year period starting in 2015, regular capture visits of barred grass snakes (*Natrix helvetica*) were made at a private site consisting of mixed wetland habitat in Norfolk, England. These surveys were conducted using artificial cover objects, in order to assist in the reliable and repeatable detection of snakes. Over this time, 1934 capture events were recorded from 1143 individual snakes. Use of ventral scale pattern variation and the software Wild-ID enabled identification of individual snakes, and therefore their recapture with high confidence across the study period. Despite the fact that the five separate observers who completed the captures (at different periods of time) had different levels of experience, the sex ratio of captured individuals was consistent through time. Stages of the slough cycle were only recorded during the last three years of data collection, but were also consistent between years over this period. While significant variation in size class and scaled mass index occurred between years, since these data rely on objective measurement of body mass and length, it seems most plausible that these findings reflect natural fluctuation between years, such as seasonal flooding impacts, rather than observer bias between surveyors These results indicate that the population data obtained over the period 2019-2021 inclusive are comparable to those collected by earlier surveyors, especially those factors most vulnerable to observer bias such as sex, and the entire seven years of data can be used to model the dynamics and apparent annual survival of *N. helvetica* at the study site.

2.2 Introduction

There is a paucity of information regarding global snake population trends and the demographic drivers influencing abundance (Böhm *et al*., 2013; Saha *et al*., 2018). This knowledge gap is primarily due to the lack of monitoring data for reptiles, which stems from their low probability of detection (Durso *et al*., 2011) relative to other taxa, such as birds or mammals (Kéry & Schmidt, 2006; Moore *et al*., 2004; Wintle *et al*., 2005). For example, Kéry (2002) estimated the detectability of a Swiss population of European grass snakes (*Natrix natrix*) as 0.11–0.25 depending on the population size, whereas Kéry & Schmidt (2006) found that the mean detectability of 150 Swiss bird species was 0.89 (range 0.72–

1.00). From an ecological perspective, detectability is the probability of determining the occurrence of a target species using a particular survey method (Kéry & Schmidt, 2008). This demonstrates the large disparity between the detection probability of snakes and other taxa that are comparatively well studied.

One mechanism to increase the detectability of reptiles in temperate regions is by deploying artificial cover objects (ACOs; Gent & Gibson, 1998; Engelstoft & Ovaska, 2000). These can be objects such as corrugated metal or bitumen-based sheets that are laid on the ground, allowing animals to shelter under them (Gent & Gibson, 1998; McDiarmid *et al*., 2012). These ACOs increase thermoregulation opportunities for reptiles, while also providing protection from predators (Sewell *et al*., 2012). Artificial cover objects also act to standardise survey effort, reducing observer biases that may be incurred using techniques such as visual encounter surveys (Willson, 2016).

Previous studies have demonstrated that temperature and cloud cover can influence the abundance and capture rate of snakes thermoregulating under ACOs (Joppa *et al*., 2009; Sewell *et al*., 2012). Equally, reptiles may only use ACOs when air temperatures are lower compared to the microhabitat provided by that ACO (Lemm & Tobler, 2021). Nevertheless, as many reptiles are attracted to the thermal conditions provided by ACOs, they provide a convenient way to survey those species that might otherwise use cryptic refuges that would be inaccessible to the surveyor. However, ACOs may only be attractive to snakes in certain environmental conditions, such as being placed in suitable habitats that are exposed to the sun, and so this needs to be taken into account when planning survey work (Gent & Gibson, 1998).

There have been relatively few studies investigating the demographic drivers underpinning changes in the size of reptile populations, in comparison to other taxa such as mammals and birds (Lowe, 1969; Hoekstra *et al*., 2020). A small number of previous studies have recorded sex ratios and age structures (Blouin-Demers *et al*., 2002; Plummer, 1985) and, using these data, researchers have been able to estimate survivorship for some snake populations (Madsen, 1987; Riedle, 2014). To date there have, however, been very few studies exploring the demography of species within the genus *Natrix*, with the focus being on *N. natrix* in Germany and Sweden, rather than the UK (Madsen, 1987; Mertens, 1995). These studies from mainland Europe have found that fecundity correlates with body size, and the size class distributions of *N. natrix* are stable through time (Madsen, 1987; Mertens, 1995).

The population of barred grass snakes (*N. helvetica*) on which this study is based has been monitored since spring 2015, with five different surveyors conducting research over the subsequent seven years. Artificial cover objects combined with photographs of individual snakes were used throughout this period to monitor trends in abundance and survival (see Chapter 4). Due to the long-term nature of the ongoing monitoring, this introduced the potential for additional variability due to biases in surveyor effort or experience, or natural variation in the population. Likewise, further error may be introduced if there is inconsistency in the reliability of identification of individual snakes between observers. It was therefore important that these data were interrogated to: (1) evaluate whether image quality permitted confident individual snake identification and recapture across the study period; and (2) to compare the population structure in terms of sex ratio, size class and scaled mass index encountered each year over the study period. The outcomes of this data exploration were used to evaluate whether robust modelling of data from different surveyors (four MSc students, and one PhD student) could be performed across the seven years combined.

2.3. Methods

2.3.1 The survey site

Watermill Broad Nature Reserve, Cranwich, Norfolk (52°31′51″N, 000°37′02″E) was used as the site for this study. The reserve is approximately 110 acres (0.45 km^2) in size and contains a number of artificial water bodies created by the process of gravel extraction (the former commercial use for the study area). This has created a mosaic of habitats including reed beds, wet woodlands and grasslands, all habitats favoured by grass snakes (Gent & Gibson, 1998). Previous surveys were undertaken by individual MSc students from April-July each year from 2015 to 2018, with each student collecting a single season of data. During the course of this PhD study (2019-2021), field surveys were undertaken May-October by a single surveyor, other than when the COVID-19 pandemic precluded fieldwork (Table 2.1). The site is subject to flooding, which has led to some periods when surveys could not be conducted for access reasons, most notably in June 2016 and March to May 2021.

Table 2.1: A summary of the effort from the five different surveyors (four MSc students and one PhD student) across the seven years of capture visits, indicating the frequency of successful capture days each month compared to the total number of capture days for that period. These data are not available for 2015-2018. Capture visits conducted from 2015 until 2018 were completed by a different MSc student each year, with visits conducted by the same surveyor from 2019.

	2015	2016	2017	2018	2019	2020	2021
April	8	1	1	0	0/0	0/0	0/0
May	9	5	8	4	15/17	0/0	7/11
June	8	2	8	11	16/16	0/0	13/16
July	0	4	1	4	12/13	18/18	14/19
August	0	6	0	0	16/16	9/9	12/18
September	0	4	0	0	8/10	9/9	11/16
October	0	1	0	0	0/0	0/0	0/0
Total	25	23	18	19	67/72	36/36	57/74

2.3.2 Placement of artificial cover objects

A total of 67 ACOs were placed around the field site in suitable areas of grass snake habitat in 2015, with a further two added in 2019. ACOs were removed during the autumn/winter period, and then replaced within 2-5 m of the original locations approximately 5 weeks before monitoring commenced in the following year. The spacing between ACOs depended on the available microhabitat, but the average distance between adjacent ACOs was 51.7 m, with a mean density of 1.18 ACOs per hectare, due to the nature of the site, with large areas covered by open water (Fig. 2.1). The material chosen for the ACOs was Onduline roofing sheets, due to their availability and ease of transport. Thirty-five of the ACOs measured 1 m x 2 m, with the remaining 34 being approximately 1 m x 1 m in size: the two sizes of ACO were placed alternately throughout the study site to avoid any potential spatial biases.

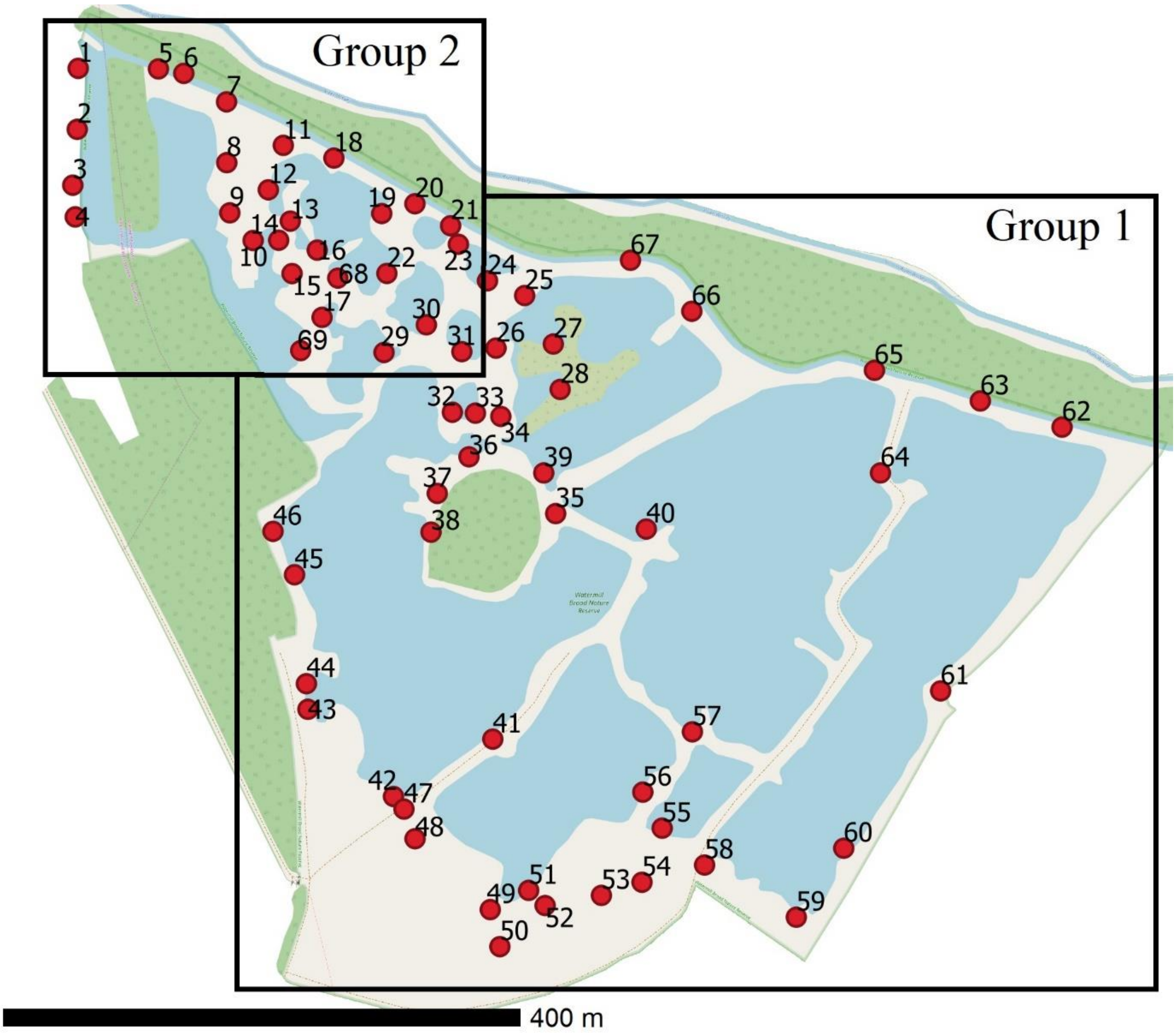


Figure 2.1: A map of Watermill Broad Nature Reserve, showing the location of the 69 artificial cover objects and the two groupings used when undertaking capture visits for barred grass snakes (*Natrix helvetica*). The two grouping areas contained equal numbers of refugia and reflect the number that could be visited within the optimal capture window, accounting for the time needed to process captured snakes. Map made using QGIS 3.12 (QGIS.org, 2020).

2.3.3 Order and timing of capture visits

Capture visits were conducted twice a day, between 0800 and 1100 hrs and 1600 and 1900 hrs during 2019 to 2021, following Gent & Gibson (1998). Prior to 2019, capture visits were undertaken multiple times a week in the morning period (described above), ending when the capture rate became too low to be productive, or when the MSc student had completed their duration of data collection. The exact start time of the morning surveys depended on the weather and time of year but were generally initiated from 0800 and 0900 hrs. As visits were timed to coincide with the presence of snakes, this was not considered to be a potential source of bias as the capture visits were not intended to act as an abundance survey.

The aim was to conduct a minimum of three surveys a week, with every ACO on site being checked on average 1-2 times a week in 2019, 1-2 times a week in 2020 and 2-3 times a week in 2021. Capture visits focussed on two non-overlapping areas (Fig. 2.1), each encompassing approximately half of the total number of ACOs. Due to the processing time of captured snakes, it was not possible to survey the entire site within either the morning or afternoon survey windows. Where possible, both groups of ACOs were surveyed on the same day; if this was not possible, either due to the weather or localised flooding, the second group was surveyed on the earliest subsequent date. At the beginning of each capture visit, the time was noted, air temperature (±1.0°C) and humidity (±5%RH) were recorded using a dual digital thermometer and hygrometer (UNI-T UT333, Uni-Trend Technology) respectively, and the wind speed (m/s) was also taken using a handheld anemometer (Proster TL0017). The optimal air temperature for snake surveys is between 12-20˚C (Gent & Gibson, 1998), the range in which individuals are active and not at risk from overheating; capture visits were avoided if air temperatures exceeded 30˚C, or were below 10 ˚C. Periods of prolonged heavy rain were also avoided but visits were conducted during light rain (rain forecast to be <4 mm per hour).

2.3.4 Capture, processing and release of grass snakes

Due to the known presence of snakes infected with the fungal pathogen *Ophidiomyces ophidiicola* (hereafter *Oo*), the causative agent of ophidiomycosis, strict biosecurity protocols were designed to limit inadvertent transmission of this, or other, pathogens when handling and processing snakes. The protocols outlined below received ethical approval from both the Durrell Institute of Conservation and Ecology and the Zoological Society of London (IOZ35).

Nitrile gloves were worn at all times and were changed between the handling of each individual snake. Upon the lifting of each ACO, the snakes underneath were caught by hand and placed into individual clear polythene freezer bags, minimising the probability of cross-contamination of *Oo* DNA between individuals. The bags were tied loosely to prevent escape but without incurring risk of suffocation, and then placed into dark cloth bags that limited light, and therefore allowed snakes to settle. If more than one snake was found under an ACO, a maximum of two were captured at a time. Snakes that were also encountered basking in the open while walking a directed transect around the site, were also captured and processed using the same protocol. When this occurred, the location where these individuals were caught was recorded using a handheld GPS (Garmin GPSmap 62st).

After 30-45 seconds in the dark cloth bag, each snake was removed and weighed whilst still in their individual plastic bag to the nearest 5 g, using a Pesola Medioline 40600 spring scale (PESOLA Präzisionswaagen AG). Snakes were photographed with the ventral surface exposed on a sheet of white acrylic, in order to best capture their anterior ventral scale pattern for use in individual recognition (Carlström & Edelstam, 1946). Each surveyor took photos on a different camera, but image quality was sufficient to not influence the identification of snakes. From 2019-2021, photographs were taken with a Pentax K-50 DLSR using an 18-55 mm lens. The first 20 ventral gular scales of each were photographed (following Dowling, 1951), but the first 16 were sufficient to allow for the identification of individuals (Baker & Allain, 2020). The length of each snake was measured using a flexible fibreglass tape to the nearest 0.5 cm, recording both snout to vent length (SVL) and tail length (TL), sex, and size class were assessed following the methodology outlined below in sections 2.3.5.1 and 2.3.5.2.

If snakes voided faeces in the process of being handled, these were collected and stored in 70% ethanol for potential future dietary analysis through the use of metabarcoding prey item DNA; opportunistic swabbing of these voided faeces was also undertaken, to screen snakes for the presence of *Salmonella* spp. through microbiological examination as part of a separate study. After snakes had been processed, they were released at the point of capture. If the snake feigned death during handling, it was placed under or near the ACO it was captured under, in order to reduce the risk of predation.

2.3.4.1 Swabbing snakes with skin lesions

While snakes were within their individual clear plastic bags, they were visually inspected for the presence of skin lesions. If present, these were swabbed in duplicate with MW-100 swabs (Medical Wire Equipment), wetted with PCR-grade water (ThermoFisher Scientific). Additionally, every fifth snake captured without detectable skin lesions was also swabbed along the ventrum to act as a control group. All swabs were then stored at -20°C until later analysis using an established real-time PCR protocol to detect *Oo* DNA. Skin sloughs and carcasses encountered during surveys were also collected, for later post-mortem examination and diagnostic testing. For more information regarding the field and lab methods, and the results see Chapter 3.

2.3.4.2 Recording behavioural responses of captured snakes

The behavioural responses that snakes exhibited during the initial capture phase were recorded, and typically comprised death feigning, hissing, and/or musking. The behavioural repertoire was then compared between snakes with and without skin lesions, to appraise any variation in predator avoidance associated with disease status. See Chapter 5 for more detail regarding the methods, and results.

2.3.4.3 Disinfection and waste protocol

After release, any equipment that had been used in the process of measuring and photographing the snakes was scrubbed with a sturdy brush to remove any mud or other detritus, prior to being sprayed and disinfected with Dettol All in One disinfectant spray (0.1 % Alkyl Dimethyl Benzyl Ammonium Saccharinate), with a contact time of 10 minutes (Rzadkowska *et al*., 2016). Once the required contact time had passed, Dettol All in One disinfectant wipes were used to wipe the surfaces clean, before being rinsed with clean water, with equipment left to air-dry in direct sunlight. All gloves and plastic-ware were disposed of as clinical waste by incineration, organised through ZSL London Zoo and an external provider.

2.3.5 General methods

2.3.5.1 Sexing methods

Two morphological features were used in combination to sex captured snakes, with one or both methods being used where necessary. Males were identified by the presence of a hemipenal bulge at the base of their tail, which is one of the most reliable ways to distinguish the sexes in adult snakes (Beebee & Griffiths, 2000), or by counting sub-caudal scales. The sex of neonates and sub-adults was determined by the number of sub-caudal scales; male *N. helvetica* have greater than 62 sub-caudal scales, whereas females have fewer than 62 (Langton, 1989; Gent & Gibson, 1998). In 4.6% of cases, it was not possible to assign a sex to an individual (Table 2.3). This was typically due to disagreements in assignment between captures, which were often caused by damage to the caudal end of the snake or the snakes not being sexually mature (i.e. sub-adults and neonates). Probing was not used, as the previous surveyors had not employed it, partly due to their differing skill levels. Given the confirmed presence of ophidiomycosis at the study site, non-invasive methods were preferred for determining sex to reduce the amount of equipment that had to be disinfected between the handling of snakes, and the risk of introducing *Oo* to the internal body cavity of snakes.

2.3.5.2 Determining the size classes of captured snakes

Wild snakes cannot be easily aged, and length is therefore often used as a proxy to allocate them to different age cohorts (Halliday & Verrell, 1988). While there have been some attempts to convert measurements to ages (Madsen, 1983), consistency between populations is unknown and a simple three-level categorisation was therefore used in this study. Mertens (1995) regards adult male grass snakes as those that have attained a SVL above 40 cm, and females with an SVL above 60 cm, consistent with Beebee & Griffiths (2000), who reported that adult males attain a minimum length of 50 cm and adult females reach a minimum length of 60 cm. For the purposes of this study, individuals of either sex measuring 50 cm or more were categorised as adults. Grass snakes with a SVL below 20 cm were categorised as neonates (Gent & Gibson, 1998; Beebee & Griffiths, 2000), while snakes exhibiting a SVL between 20 and 50 cm were categorised as sub-adults. Hatching of *N. helvetica* eggs occurs during August and September (Beebee & Griffiths, 2000), so if snakes measuring <20 cm were encountered before mid-August, they were assumed to be hatchlings from the previous season, that had overwintered with little subsequent growth due to unfavourable conditions (Madsen, 1983), and were omitted from the analysis.

2.3.5.3 Calculating scaled mass index (SMI)

The scaled mass index was used to assess the body condition of captured grass snakes, as it is better suited for this use with snakes than the more simplistic log-transformed body condition index (Peig & Green, 2009). Female barred grass snakes that were gravid were excluded from the analysis, due to their small sample size (range 1-3 per annum) and potential to bias the results. While the presence of a food bolus could represent another potential confounding variable, snakes with an apparent recent meal were retained within the dataset. This approach was taken for consistency since data on food bolus detection were only available for the 2019-2021 surveys, therefore, it was not possible to account for this feature across the study period. Given how frequently snakes with a recent meal present were encountered from 2019 onwards (45.2% of captures), it is very likely that a large number of snakes that were historically encountered also had a food bolus present.

2.3.5.4 Identification of snakes using Wild-ID

Photos of the ventral scale patterns of the first 20 scales of each snake (see Section 2.3.4), were straightened in the open source image manipulation software I^{3}S-Straighten (Den Hartog & Reijns, 2015; Rosa *et al.*, 2020). This allowed for the comparison of photos using the open source pattern-matching software Wild-ID (Bolger *et al.*, 2012). Wild-ID uses the

Scale Invariant Feature Transform (SIFT) operator when matching pairs of images; the SIFT operator was designed to find and extract distinctive image features invariant to image scale, rotation, viewpoint, local distortion and illumination (Lowe, 2004). This makes Wild-ID ideal for the matching of *N. helvetica* ventral scale patterns, given that multiple surveyors were involved throughout the duration of the project, each with different digital or phone cameras.

For each target image loaded into Wild-ID, the user interface presents the 20 most similar pre-existing images in rank order according to the matching score on a scale of 0-1, where 1 is a perfect match. Wild-ID displays the target image and each match side-by-side, allowing for a direct comparison between the two. If the ventral scale pattern visually matched that of a pre-existing image within the database, then it was considered to be that individual snake captured at a later point in time. If no matches were found among the top 20 ranked images, the snake was assumed to be an individual not previously encountered from any of the field seasons.

All of the images collected from 2015 to 2018 were additionally matched manually, to establish the mismatch rate before the standardisation of ventral pattern photographs was introduced in 2019. This was also completed to ensure a consistent image classification system could be applied to the images taken by the previous four surveyors. To identify the mismatch rate of ventral scale patterns following the introduction of a standardised photographic protocol, a subsample of those images from 2019 onwards where snakes had distinctive markings or other potentially identifying features (n = 33), were manually checked against the library of previously encountered snakes to screen for a match.

2.3.5.5 Assessment of slough cycle stage

Sloughing occurs twice yearly in males, once yearly in females, and more frequently in growing sub-adult snakes (Beebee & Griffiths, 2000). Other factors such as the temperature and the size of snakes, may also influence how frequently they slough (Semlitsch, 1979). It was important to determine the sloughing phase each snake was in at the point of capture, as this may influence the occurrence and detectability of skin lesions (Chapter 3). From 2019, a modified version of Maderson's (1965) protocol was used to determine the slough cycle of captured *N. helvetica* (Fig. 2.2), excluding stages dependent on characteristics that could not be easily determined in the field. Phase 0 snakes were defined as those that had recently sloughed, with glossy black markings on their ventral scales (Stage 1 in Maderson (1965)); Phase 1 snakes were those exhibiting visible greying of the ventral scales where they approached sloughing (Stage 3 in Maderson (1965)); and Phase 2 snakes were those with cloudy eyes where sloughing was imminent (Stage 4 in Maderson (1965)). The presence of a lymph-like fluid that builds up between the old and new layers of skin immediately before sloughing, gives them a clear appearance, which can also appear glossy and result in the miscategorisation of snakes that are about to slough, as freshly sloughed individuals (i.e. incorrect classification of Phase 2 as Phase 0). However, snakes only remain in this state with a clear scale appearance for five to eight days until the snake sloughs (Maderson, 1965), so the potential for error is low. Snakes may remain in Phase 0 for periods of weeks or months, depending on size, food availability, and temperature, and Phase 1 for a considerably shorter period of time (Maderson, 1965).

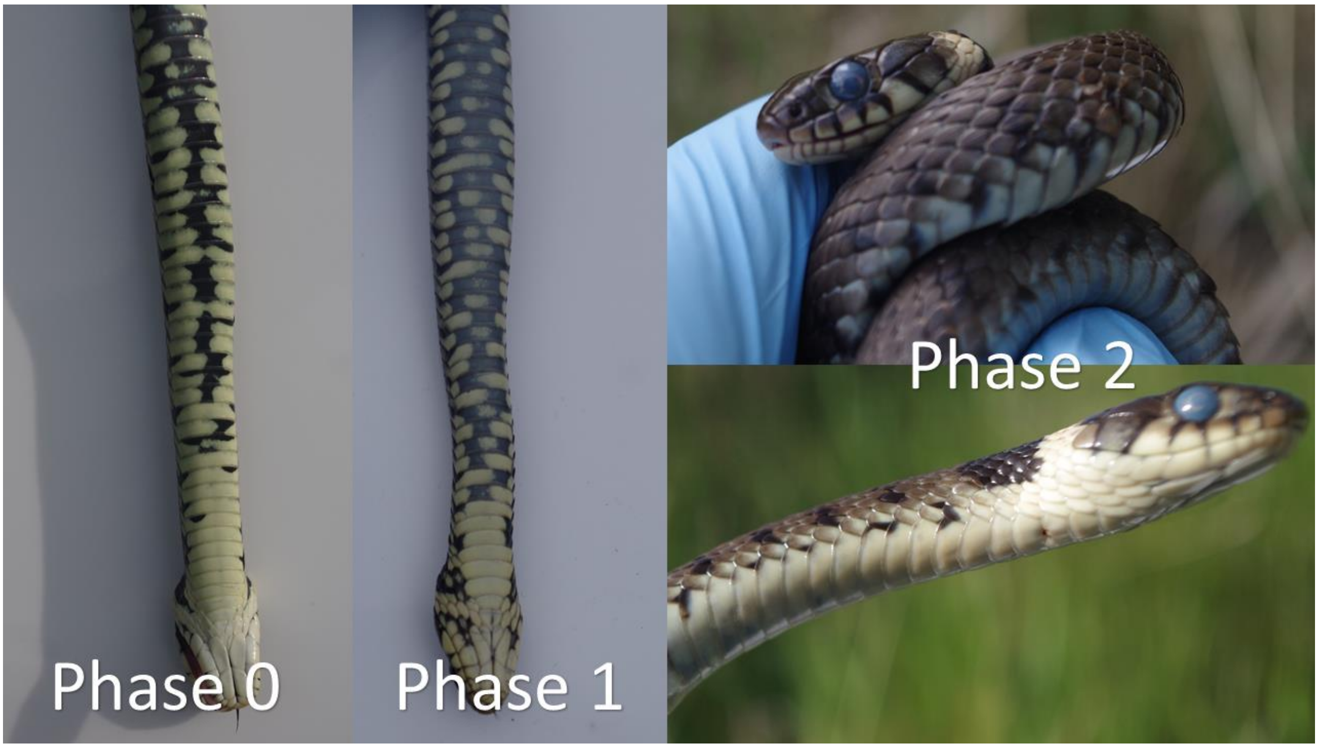


Figure 2.2: Images showing differences between each of the three phases of the sloughing cycle in the barred grass snake (*Natrix helvetica*). Note the dulling of the black ventral scale pattern in Phase 1, compared to Phase 0.

2.4 Results

2.4.1 Captures

The numbers of surveys undertaken varied each year, primarily due to the constraints imposed on MSc student availability by their respective courses, which truncated the catching season in 2015, 2017, and 2018 (Table 2.1). The 2020 field season was similarly truncated, albeit at the start of the season, by movement restrictions imposed as a result of the global COVID-19 pandemic. The seven years of capture visits generated a combined total of 1934 encounters, averaging 276 a year (Fig 2.3). The use of ACOs proved extremely effective, with the majority of captures (97.7%, n = 1890) involving snakes found underneath them. On average 33.2% (28.5%-42.2%) of snakes encountered each season were recaptures (Fig. 2.4), of which 15.7%-35.5% involved within-season recaptures (Table 2.3).

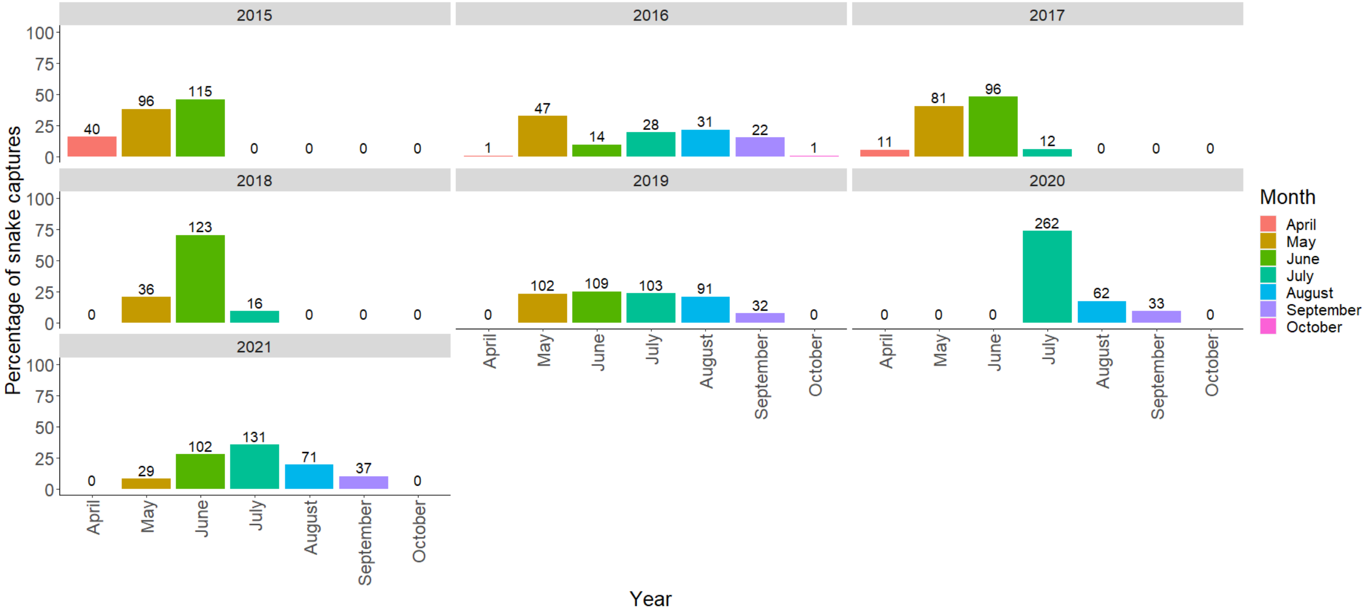


Figure 2.3: Percentage of captures of barred grass snakes (*Natrix helvetica*) per annum at Watermill Broad Nature Reserve by month; monthly totals presented above bars. The modal month for captures of snakes was June for most years, except in 2016 when the site was flooded, and in 2020 as an impact of the COVID-19 pandemic. In those years where monitoring continued after this time period, a seasonal pattern of decline in captures can be observed.

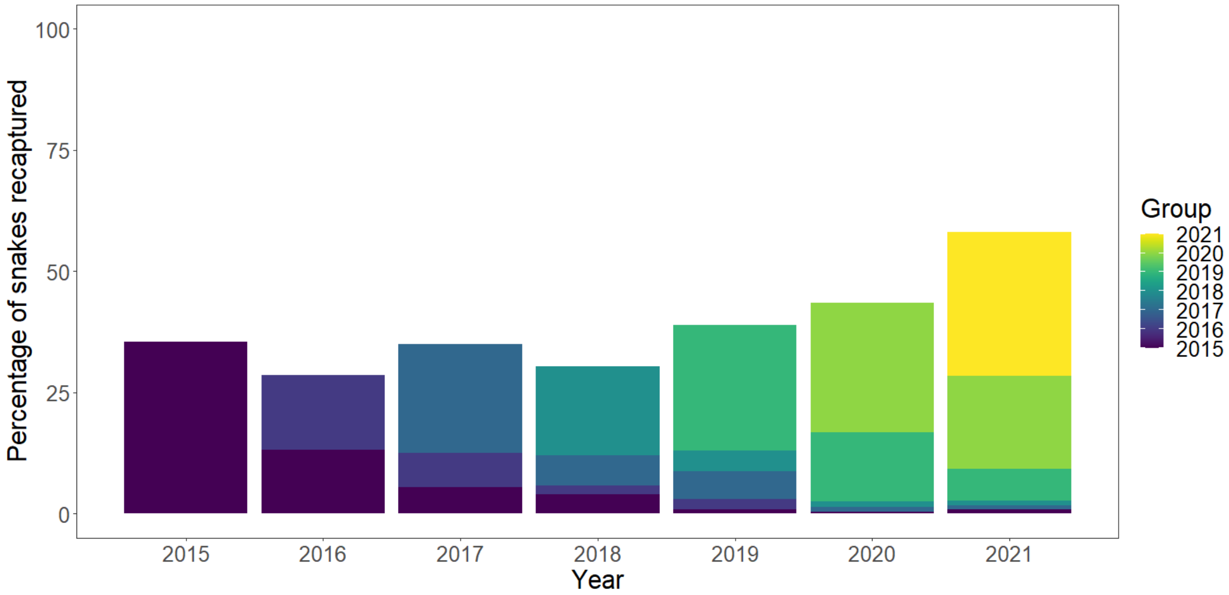


Figure 2.4: The percentage of snakes recaptured each field season (including multiple recaptures of the same individual), from 2015 to 2021, with the recaptures from each annual

capture group represented by a different colour. These data represents the percentage of recaptures from the total number of captures each year.

Table 2.2: The frequency of within-season (italics) and between-season recaptures of barred grass snake (*Natrix helvetica*) annual capture groups, across the seven years of capture data. The percentage of within-year recaptures have been provided in brackets, as a proportion of all captures that season.

	2015	**2016**	**2017**	**2018**	**2019**	**2020**	**2021**
2015	*89* (35.5%)	19	11	7	4	41	3
2016	-	*22* (15.7%)	14	3	9	90	0
2017	-	-	*45* (22.5%)	11	25	4	3
2018	-	-	-	*32* (18.3%)	19	4	4
2019	-	-	-	-	*113* (25.9%)	51	24
2020	-	-	-	-	-	*95* (26.6%)	71
2021	-	-	-	-	-	-	*110* (29.7%)
Total	251	144	200	175	437	357	370

2.4.2 Sex of captured grass snakes

The sex ratio of captured *N. helvetica* has been consistent through time at near unity (Fig. 2.5), with no significant differences in the sex ratio seen during the seven years of data collection and the five observers, when excluding those snakes where no sex was assigned, $\chi^2 = 3.36$, df = 6, p = 0.76. When assuming that all of the snakes that had an unknown sex were male, there was still no significant difference between the sex ratio seen across the seven years, $\chi^2 = 6.78$, df = 6, p = 0.34. The same was true under the assumption that these snakes may have been female, $\chi^2 = 1.69$, df = 6, p = 0.95.

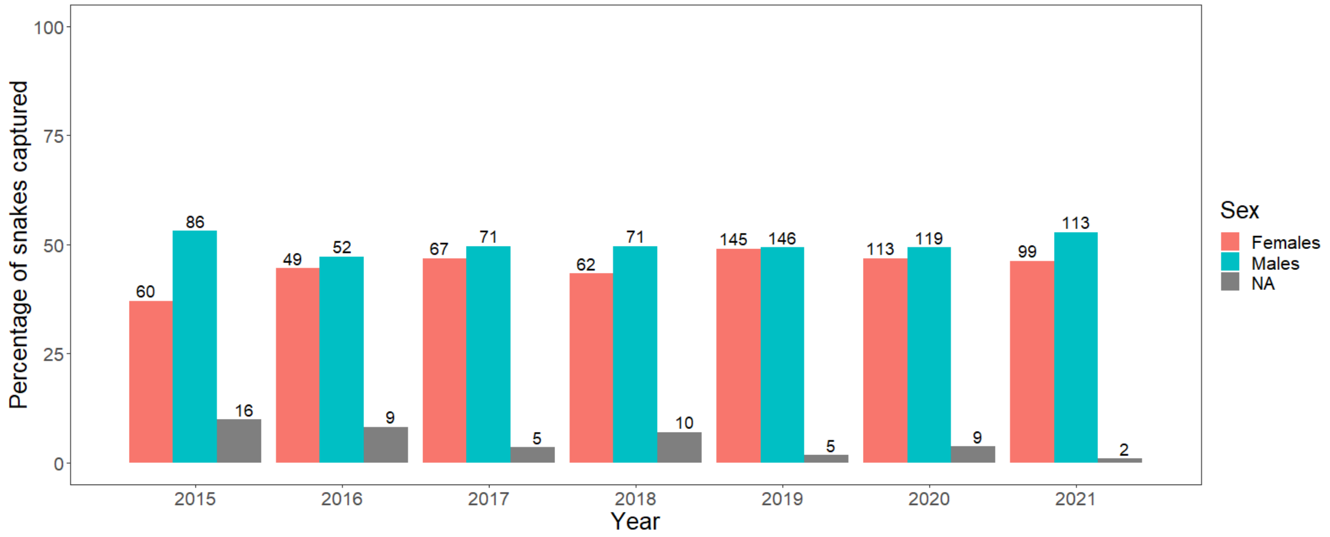


Figure 2.5: The sex of individual captured barred grass snakes (*Natrix helvetica*) 2015 – 2021, as a percentage of the overall individuals captured. Numbers over bars represent the total number of snakes in each category.

Table 2.3: The percentage of barred grass snakes (*Natrix helvetica*) captured in each year where the assignment of sex differed from that assigned on first capture. Total sample size of overall captures each year is given in brackets.

Year	**2015**	**2016**	**2017**	**2018**	**2019**	**2020**	**2021**
Occurrences	5.9% (251)	2.8% (144)	2.5% (200)	5.7% (175)	1.1% (437)	0.6% (357)	0.5% (370)

2.4.3 Size class of captured grass snakes

Sub-adults accounted were the modal size class for four of the study years (Fig. 2.6), three of which were where the observer was the same. A significant difference between the size classes of snakes captured was observed when comparing the data collected each year to one another, $\chi^2 = 53.89$, df = 6, p = <0.01, when excluding neonates and snakes with no defined size class. Neonates were excluded as capture visits only extended into September, when neonates were more likely to be observed, during four of the seven years. A comparatively lower number of adults encountered in 2016 may have been a consequence of the flooding that occurred during the middle of the field season.

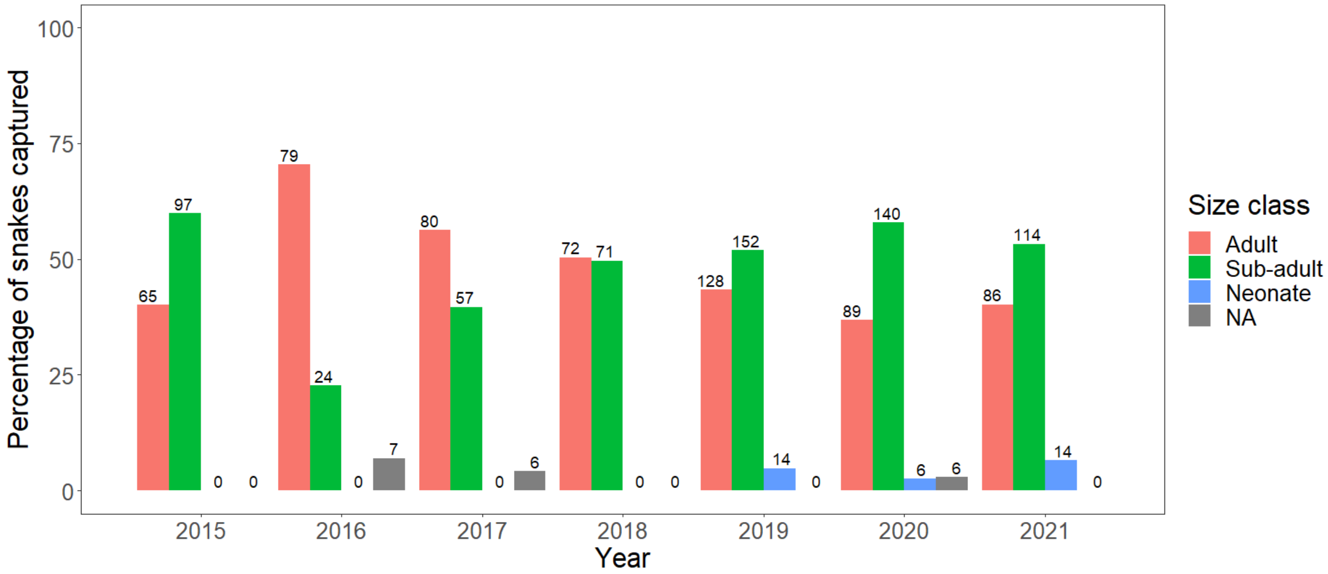


Figure 2.6: The percentage of individual captured barred grass snakes (*Natrix helvetica*), in each of the different size classes across the seven years of capture data. Numbers over bars represent the number of snakes in each category.

2.4.4 Scaled Mass Index

There was evidence of significant variation in the SMI of individuals across the seven year study period (ANOVA, F(1,1900) = 8.98, p = <0.01). However, the data were consistent for five of the seven years, with the anomalous years being 2016 and 2017, with a mean SMI of

40.54 or 130.0 respectively, compared to the range of 58.77 – 61.71 from the other five years. When these two years are removed from the analysis, there is no significant difference between the SMI of snakes captured each year (ANOVA, F(1,1580) = 0.09, p = 0.76).

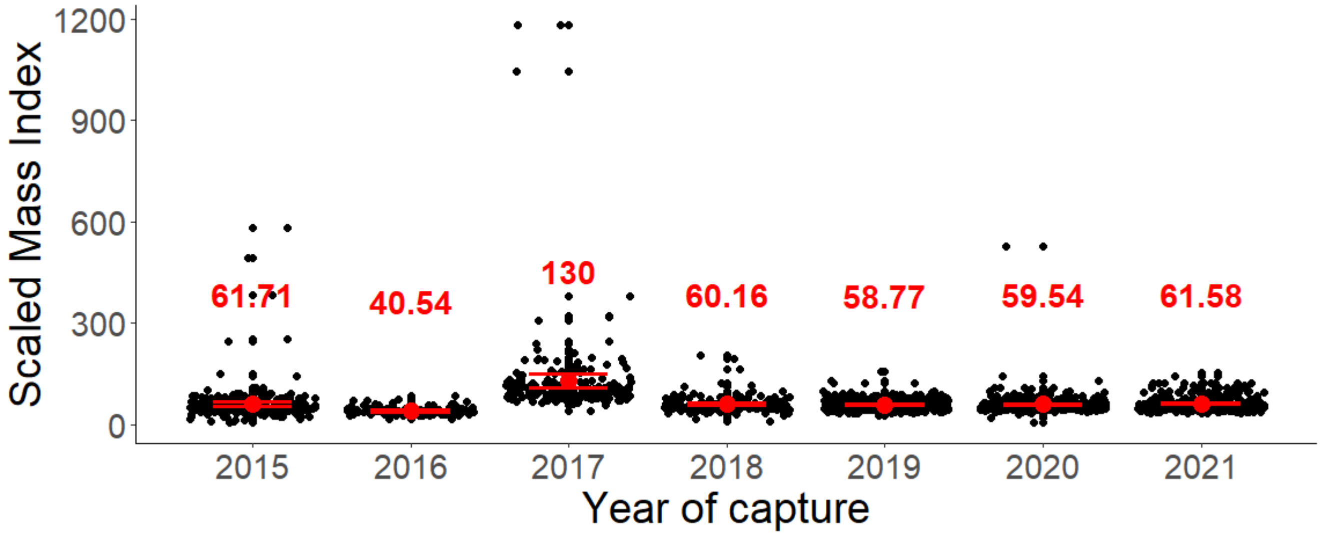


Figure 2.7: The Scaled Mass Index of captured barred grass snakes (*Natrix helvetica*) for each year from 2015 to 2021. Red dots represent the mean (value indicated in red font above), with 95% confidence bars.

2.4.5 Photo matching using Wild-ID

Wild-ID proved an extremely reliable method of individual identification of *N. helvetica*. The manual identification of images from snakes captured from 2015 to 2018 identified a mismatch rate of 1.6%. Wild-ID was also able to confirm the mismatch from previous surveyors, although it was unable to match a small number of photos (n = 5) where the snake was too coiled in the original photograph to allow the image to be straightened, or due to images being out of focus.

The mismatch rate for snakes caught over the period 2019-2021 was 0%, due to the standardised photographic protocol, based on the results of the sub-setting described in 2.3.5.4 using a sample size of 33 individuals. More than 77% of the matched snakes corresponded with the most highly ranked image using the SIFT operator (Fig. 2.8). On rare occasions (n = 12), some individuals were mislabelled as being separate individuals, but later identified as being the same snake following a subsequent match from a recapture that had two unique IDs or more. When this occurred, the two were merged. These instances only occurred within the historic dataset. This indicates that matches were not always within the top 20 ranked matches, as provided by Wild-ID but mismatches can be reduced by a standardised protocol.

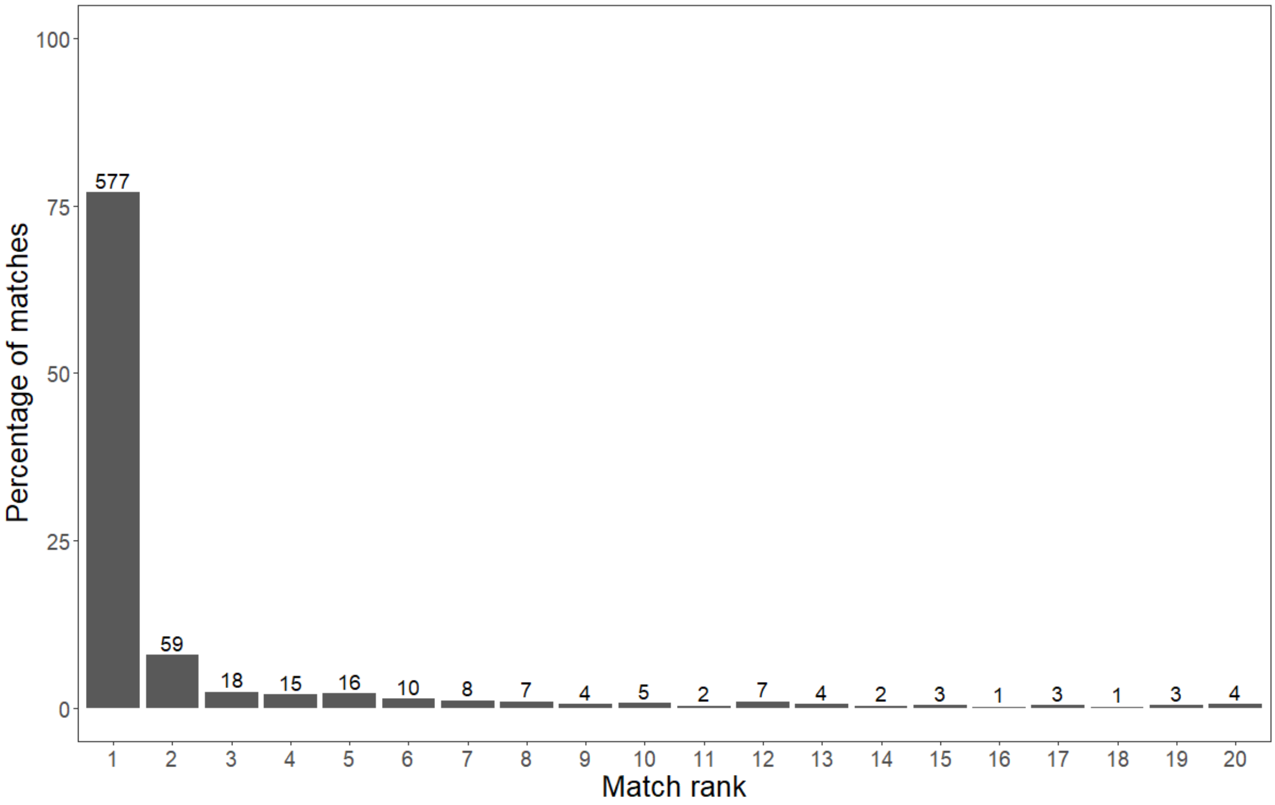


Figure 2.8 The percentage of ventral scale pattern matches in the barred grass snake (*N. helvetica*) ranked by match number in Wild-ID, for those individuals where paired scale patterns were found for all individuals encountered between 2015 and 2021.

Furthermore, 96% of matches were made within the top 10 potential matches presented by Wild-ID. The average score provided by Wild-ID (ranging from 0-1) was 0.137, with higher ranked matches having a considerably score (Fig. 2.9). The presence of skin lesions and the various stages of the slough cycle did not affect the ability of Wild-ID to match images of the same individual through time.

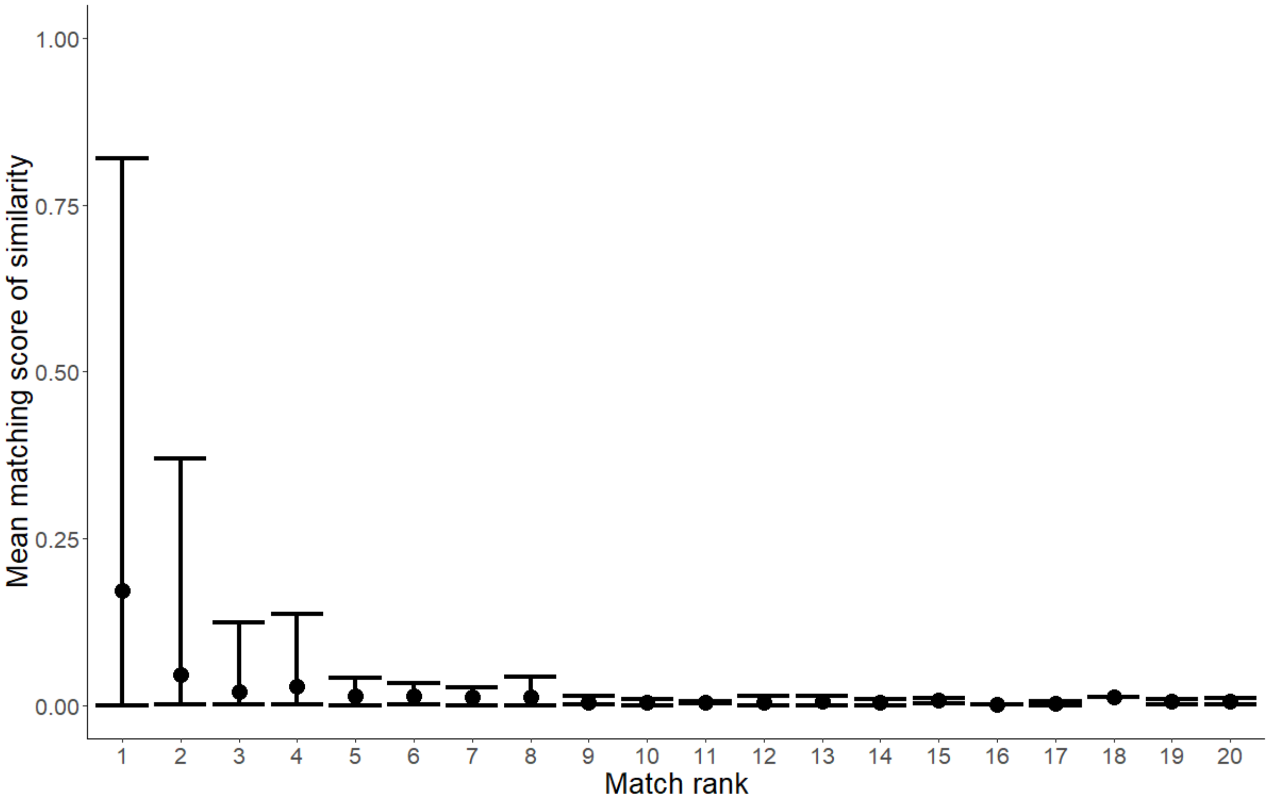


Figure 2.9: The mean matching score of similarity for each of the matched pairs of images of barred grass snake (*Natrix helvetica*) ventral scale patterns (solid black dots), with the range also displayed by the bars.

2.4.6 Slough cycle of captured grass snakes

The most frequently encountered phase of the slough cycle observed was Phase 0 (Fig. 2.10), which is the phase that snakes spend most of the slough cycle in, prior to shedding. There was no significant difference between the numbers of snakes within the three cycles across the years 2019-2021, despite the 2020 sampling season being truncated due to the COVID-19 pandemic, $\chi^2 = 8.86$, df = 2, p = 0.64.

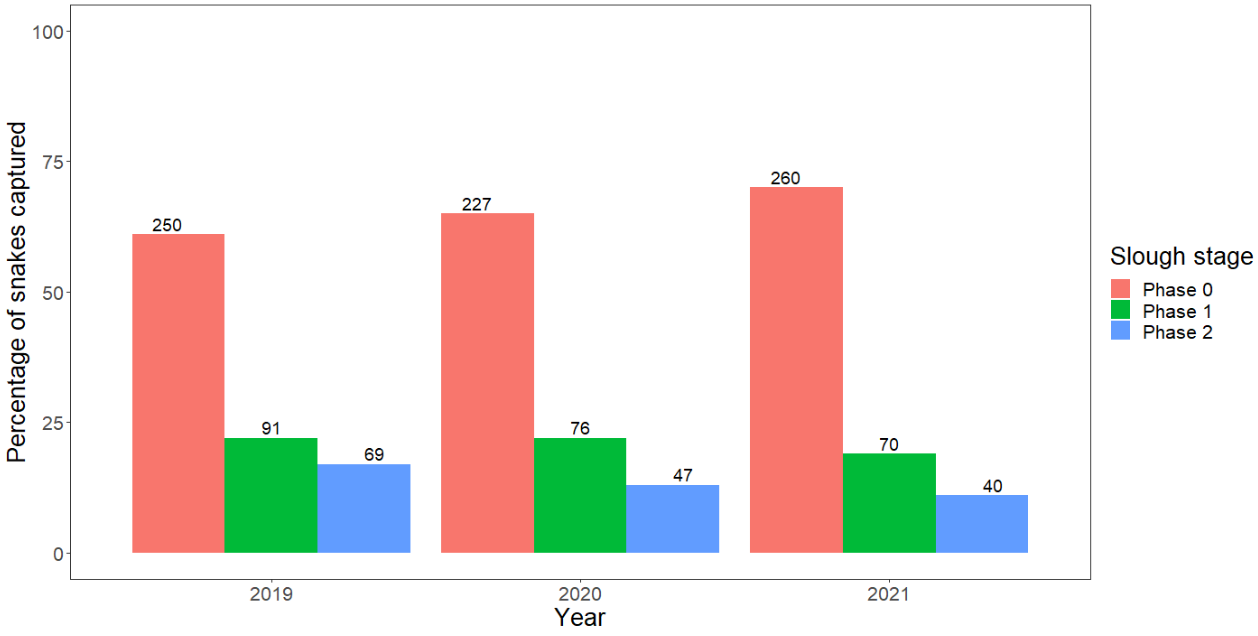


Figure 2.10: The percentage of captured barred grass snakes (*Natrix helvetica*), in each of the different slough phases. Numbers over bars represent the number of snakes in each category.

2.5 Discussion

Across the seven years of data, 1934 capture events were recorded, involving 1143 unique individuals, with photos of the ventral scale markings of snakes allowing for this individual recognition through time. Although these data were collected by five different surveyors, no significant differences in the sex ratio of snakes were found when comparing the earlier data to that collected in 2019-2021. There was some variation in size class of captured individuals, and their SMI, but when the anomalous two years were removed from the analysis, no significant difference was found. These two variables are based on objective measurements that are less likely to incur observer bias, and so the interannual fluctuations in these likely reflect variation in the population. After considering the local environmental factors that may influence the use of the site by *N. helvetica*, the percentage of within-year recaptures was also generally consistent between years. This consistency supports robust use of the pooled data to produce models on apparent annual survival in the population of *N. helvetica* studied.

Wild-ID was a reliable tool in the identification of individual *N. helvetica* caught over the seven year study period, despite a wide variation in factors such as lighting, focus, and resolution of these historic images. Wild-ID has been used in similar studies for the identification of unique individuals in a range of taxa including amphibians and other reptiles (Bendik *et al*., 2013; Suriyamongkol & Mali, 2018). A comparison between Wild-ID and other pattern-matching algorithm software packages shows that Wild-ID is better suited to some species than others such as *Bombina variegata*, and performs best with a medium to large (>2000) database of images (Matthé *et al*., 2017). This made it ideal for use with the *N. helvetica* ventral scale photos given that almost 2000 photos had been taken, since the beginning of the project in 2015.

The matching ranks plateaued after the 10th rank, indicating that there may be some individuals that may have matched previous images, if additional ranks beyond 20 had been presented (Fig. 2.8); this is one limitation of Wild-ID. However, the number of these individuals with matching images past the 20th rank is likely small compared to those presented. Any subsequent recaptures of these snakes, would have been identified, as they would have likely matched a higher ranking photo, especially following the introduction of the standardised protocol. When matching the ventral scale patterns manually by eye, there is also the risk of mismatching individuals. However, mismatches within the dataset and through the use of Wild-ID were very low. Importantly, there is no evidence to indicate a systematic discrepancy between snakes with skin lesions and those without since the presence of skin lesions did not affect the ability of Wild-ID to be able to match the ventral scale patterns of *N. helvetica*. Wild-ID was able to match photos of 52 individual snakes with and without skin lesions on serial recaptures on multiple occasions.

The seasonal pattern of captures was also variable throughout each of the study years with June being the modal month, except for 2016 due to seasonal flooding, and 2020 due to restrictions imposed by the COVID-19 pandemic (Fig. 2.3). As surveys did not start until the first week of July in 2020, it is likely the only major impact the pandemic had was on data quantity, as the data are comparable regarding the sex ratio and SMI, and slough cycle to both 2019 and 2021.

The sex ratio reported from previous studies of *N. helvetica* and *N. natrix* at other sites throughout Europe is 1:1 (Mertens, 1995; Bury *et al*., 2020). By reviewing the data collected at Watermill Broad Nature Reserve, the sex ratio was consistent through both time and with these previously studied populations, being approximately 1:1 (Fig. 2.5). This is true even when accounting for those snakes where no sex was able to be assigned to them. Recapture data supported accurate sex identity in 94.1-99.5% of snakes per year, which further builds confidence in the results across the five different observers.

Size categories were variable over time, and showed significant differences when compared between years and observers (Fig. 2.6). As these were based on objective measurements, there are unlikely to be errors in the measurements of snakes due to observer error. However, some of the variability seen throughout the seven years may be linked to observer bias, as snakes can still be cryptic when basking underneath ACOs, especially when debris and dead grass is present underneath, leading to potential detectability issues. Larger snakes are more easily detected than smaller ones, especially when coiled in the open (Lock & Griffiths, 2022). Seasonal flooding in 2016 may have reduced the number of potential egg laying sites, or may have even swamped those eggs that had already been laid, leading to a lag in recruitment that year, and reduced numbers of sub-adults in subsequent years. The flooding may have also displaced snakes that were forced to establish home ranges elsewhere, reducing the encounter rate of sub-adults during 2016.

The scaled mass index for captured individuals was significantly different through time, likely due to variation in 2016 and 2017. This fluctuation in SMI observed may have been influenced by the flooding that occurred at that time. The large discrepancy in SMI observed in 2017 could also be data collection or input errors, arising from the previous MSc student. From 2018 though, the mean SMI was more stable and comparable to that from 2015. It is hard to draw conclusions from this without a longer time scale of data, but there may be natural changes in the scaled mass index through time at the population level, as different individuals utilise the study site. Additionally, there may be climatic and dietary reasons for the variation observed between the years too, as has been observed in other snake species (Lindell, 1997).

The majority of snakes captured were in Phase 0 of their slough cycle, which reflects the longer duration in this phase, from between a couple of weeks to a couple of months depending on variables such as age and temperature (Maderson, 1965). Smaller numbers of snakes were observed during Phases 1 and 2, corresponding to the relatively short time snakes are expected to spend in each of those phases. The timing of changes between the two phases was largely synchronous throughout the three years where slough cycle was recorded, therefore could later be used in the modelling analysis. This understanding of the seasonality of shedding helped inform when searches for sloughs under ACOs would have greatest likelihood of success. These slough samples were used for *Oo* surveillance, as described in Chapter 3, and have been retained at -20 ^{o}C for potential future population genetic analyses (Jones *et al*., 2008).

In conclusion, multiple surveyors were able to catch over 1000 individual *N. helvetica* through almost 2000 capture events, across the seven years that capture visits were conducted. WILD-ID was an effective means of determining the identity of individual grass snakes and recapture rates were similar across surveyors. Some features of the population structure, such as sex ratio, were highly consistent through time. There was some variability in the effort and experience between surveyors, but this did not translate into detectable biases in the data. Appraisal of these combined analyses provide confidence in the data quality collected across surveyors, and support use of the combined seven-year dataset for population modelling, see Chapter 4.

2.6 Acknowledgements

We would like to thank Angela and Louisa Knapp for allowing us continued access to Watermill Broad Nature Reserve, the previous four Masters students (Sarah Gelpke, Angela Winnett, Helena Marsh-Williams, and Sargai Sha) from the University of East Anglia (UEA), and their supervisor Dr Iain Barr. Additionally, thanks must also be paid to Dr John Baker for his assistance in establishing this long-term monitoring project at Watermill Broad Nature Reserve, and for training the previous students how to conduct reptile surveys necessary in the historic data collection.

Chapter 3. Characterisation, prevalence and severity of skin lesions caused by ophidiomycosis in the barred grass snake (*Natrix helvetica*)

3.1 Abstract

Ophidiomycosis is an emerging infectious disease affecting wild snake populations in Asia, Europe, and North America. Recently confirmed in Great Britain, the prevalence, severity and significance of ophidiomycosis have yet to be characterised in free-living snakes at the population level in Europe. Therefore, a population of barred grass snakes (*Natrix helvetica*) in Eastern England was monitored for three seasons (May to October) from 2019 until 2021, to investigate the occurrence and characteristics of skin lesions and their aetiology. Skin lesions were present in 25.5% (191/750) of snakes encountered over the three year period, 2019-2021 inclusive. The most frequently observed skin lesion characteristics were crusting, discolouration, and scale margin erosion. The majority of skin lesions (96.9%; 185/191) were observed on the ventral surface of affected snakes, with them being present along the length of the body. The severity of the observed skin lesions was also assessed with more than half of cases (53.1%; 98/191) being mild. Skin lesions were predominantly observed in adult snakes (72.8%; 139/191). A higher occurrence of trauma was observed in snakes with skin lesions, indicating potential changes in behaviour leading to increased conflict with predators. Of the swabs taken from snakes with skin lesions (including recaptures), 78.7% (215/271) tested real-time PCR positive for the presence of *Ophidiomyces ophidiicola* DNA. Combined with histological examinations of skin lesions from sloughs and carcasses examined post mortem which diagnosed ophidiomycosis, these findings indicate that skin lesions can reasonably be used as a proxy of this disease in *N. helvetica*. Multivariate analysis determined that month of capture, sex of snakes, slough cycle stage, snout-to-vent length, and the presence of trauma were likely predictors of the presence of skin lesions in *N. helvetica*. This study will help to inform other similar research projects across sites and species in Europe, by providing a framework to determine if the characteristics associated with ophidiomycosis outlined within are representative across wild snake populations across the continent.

3.2 Introduction

In recent decades, a number of novel infectious diseases have emerged that threaten wildlife health, many of which are caused by fungi (Fisher *et al*., 2012; Fisher *et al*., 2016). The emerging fungal pathogen *Ophidiomyces ophidiicola* (*Oo*) causes the disease ophidiomycosis (commonly known as snake fungal disease or SFD) in infected snakes, recorded from wild populations in both North America and Europe, being first detected in 2008 and 2010 respectively (Franklinos *et al*., 2017; Lorch *et al*., 2016). *Ophidiomyces ophidiicola* was confirmed as the causative agent through fulfilment of Koch's postulates (Lorch et al., 2015). Through the analysis of museum specimens, *Oo* has been demonstrated to have been present in wild snakes in the USA since at least 1945 (Lorch *et al*., 2021). In 2019, cases of ophidiomycosis were confirmed from wild snakes in Asia for the first time (Grioni *et al*., 2021; Sun *et al*., 2021). As well as wild snakes, there have also been confirmed detections of *Oo* in captive snakes with skin lesions from Asia, Australia, North America, and Great Britain (Sigler *et al*., 2013; Takami *et al*., 2021). Some of these samples taken from captive snakes date back to 1985, predating many of the wild snake detections (Sigler *et al*., 2013).

Ophidiomycosis was first diagnosed in the barred grass snake, *Natrix helvetica,* in Great Britain through examination of carcasses and skin sloughs, supported by ancillary diagnostic testing (Franklinos *et al*., 2017). These samples were collected opportunistically through a national disease surveillance program, with a widespread geographic distribution in southern and central England. The presence of *Oo* was confirmed using real-time PCR (rtPCR) (Bohuski *et al*., 2015), fungal isolation and sequencing, and ophidiomycosis was diagnosed through histopathological examination (Franklinos *et al*., 2017). The timeline of these positive samples dated back to the start of the available slough archive (2010-2016). The disease may have been present for some time before then and previously missed in Great Britain, rather than representing a recent emergence. It is not known whether *Oo* is an introduced, or an endemic pathogen, in Great Britain (Franklinos *et al*., 2017). Recent analysis of museum specimens has indicated that *Oo* has been present in *N. helvetica* since at least 1959 in mainland Europe (Origgi *et al*., 2022).

A single skin slough with macroscopic lesions from the Czech Republic from a dice snake (*N. tessallata*) also tested PCR-positive for *Oo* DNA (Franklinos *et al*., 2017). There has been an additional report of a case of ophidiomycosis from Switzerland in the European grass snake (*N. natrix*) with crusted skin lesions from which *Oo* was detected by rtPCR (Meier *et al*., 2018). All three of these *Natrix* species have a similar ecology, being semi-aquatic predators of amphibians and fish, which may make them more susceptible to infection due to their use of both terrestrial and aquatic habitats. Alternatively, diagnosis in these species may be a consequence of their abundance and wide geographical range, so this pattern may simply reflect observer bias. Finally, a single rtPCR-positive adder (*Vipera* *berus*) from Great Britain, with no detected skin lesions, represents the fourth species from which *Oo* has been detected from Europe: this may represent either carriage, early infection or environmental contamination (Franklinos *et al*., 2017).

In North America, where *Oo* has been studied most extensively, there is evidence to suggest that the fungus has a wide taxonomic and ecological range of potential snake hosts (Burbrink *et al*., 2017). The same is likely true in Europe, despite a current lack of published evidence, given the similarities of taxonomic and ecological diversity between the snakes in North America and Europe. Whether the smooth snake (*Coronella austriaca*), the third native species in Great Britain, is susceptible to ophidiomycosis remains unknown.

The clinical signs of ophidiomycosis vary depending on the severity of infection and how deep *Oo* has penetrated within the infected snake's tissues. Clinical signs may also differ between snake species (Lorch *et al*., 2016; Franklinos *et al*., 2017). The most commonly observed indications are skin lesions, which may take the form of crusting, erosion and ulceration (Lorch *et al.*, 2016). In some cases, nodules or granulomas may develop when deeper tissues have been invaded by the fungus (Lorch *et al.*, 2016). There has been some attempt to score the severity of infection based on these clinical signs in North America (Baker *et al*., 2019; McCoy *et al*., 2018), however such work has yet to be conducted in Europe. The clinical signs may vary between these continents due to the existence of two clades of *Oo* detected in wild snakes from North America and Europe, with distinctive genetic and phenotypic characteristics (Franklinos *et al*., 2017). It is not clear whether the identified lineages of *Oo* in North America and Europe have different rates of transmission, virulence or clinical signs (Franklinos *et al*., 2017). These potential differences may mean that scoring schemes devised for snakes in North America are not applicable to those in Europe, and vice versa.

Ophidiomycosis has the potential to impact wild snake populations by causing morbidity and/or mortality (Allender *et al.*, 2015; Lorch *et al.*, 2015). Subsequent secondary infections, such as those by the fungus *Fusarium* spp., may also lead to mortality or morbidity in snake populations (Barber *et al*., 2016). In addition to these direct causes of mortality attributed to ophidiomycosis, indirect mortality may also occur as a result of *Oo* infection. For example, changes to behaviour such as basking in more open areas following infection, thereby increasing the body temperature of snakes in order to promote their immune function to assist in clearing infection, may make snakes more vulnerable to predation or exposure to temperature extremes (Lorch *et al*., 2015; Tetzlaff *et al*., 2017). Evidence from challenge studies suggests that experimentally infected corn snakes (*Pantherophis guttatus*) may suffer from bouts of anorexia (Lorch *et al*., 2015). An increased rate of sloughing has also been observed in infected snakes, which may be associated with an elevated energetic cost (Lorch *et al*., 2015). Despite these behavioural changes, current evidence from monitoring studies of eastern Massasauga rattlesnake (*Sistrurus catenatus*) populations suggests that the relationship between body condition index and ophidiomycosis is variable (Allender *et al*., 2016; McCoy *et al*., 2017).

Barred grass snake monitoring was established at a site in Norfolk (Watermill Broad Nature Reserve, Cranwich) in 2015, through collaboration between the British Trust for Ornithology (BTO) and students at the University of East Anglia (UEA). This study had an initial focus on environmental drivers of annual variation in phenology and abundance, and the demographic mechanisms underpinning them. Ophidiomycosis was first confirmed at the site in 2016, through opportunistic sampling of carcasses, skin sloughs and swabbing of skin lesions by Angela Winnett, an MSc student from UEA (Winnett, 2017), one of the first records of the disease for this species in Great Britain. As a result of this finding, intensive surveillance to investigate the occurrence of ophidiomycosis in this grass snake population was conducted over three consecutive seasons of snake activity, 2019-2021.

Using standardised survey protocols, the aims of this research were to: (1) investigate the character, distribution, and severity of skin lesions; (2) assess whether those skin lesions were caused by ophidiomycosis using rtPCR and histology; (3) determine the seasonality of skin lesions; (4) ascertain the size class and sex of snakes with observed skin lesions; (5) establish if trauma was associated with the presence of skin lesions; (6) test whether the body condition of snakes differed with skin lesion presence; (7) compare the character and severity of skin lesions between serial recaptures to assess progression or recovery of skin lesions over time; and finally, (8) to determine whether the presence of skin lesions is correlated with factors comprising sex, snout-to-vent length, slough cycle, presence of trauma, presence of a food bolus, and month of capture.

3.3 Materials and methods

3.3.1 Systematic field surveys and sample collection

Structured surveys were used to detect and capture individuals of *N. helvetica* at the study site, with 69 artificial cover objects (ACOs) placed at approximately regular intervals (between 18.0 m and 238.6 m apart, 51.8 m on average) in potential grass snake habitat (For more information on the methods used, see Chapter 2). Briefly, ACOs were checked on average 1.7 times a week in 2019, 1.7 times a week in 2020 and 2.8 times a week in 2021. Morning surveys were conducted between 0900 and 1100 hrs and afternoon surveys between 1600 and 1900 hrs, when weather conditions allowed. When snakes were encountered while basking the open, these were also captured, comprising a minority of samples collected.

The protocol for snake capture and handling received ethical approval from both the Durrell Institute of Conservation and Ecology (5-PGR-18/19) and the Zoological Society of London (IOZ35). When snakes were encountered, they were captured by hand and placed in individual transparent plastic bags. Visual inspection of the entire body was conducted for evidence of skin lesions for each snake. Body length and body mass were recorded using a tape measure (to the nearest 0.5 cm) and Pesola spring balance (to the nearest 5 g). Snakes were sexed based on the length and profile of their tail; individuals with a noticeable vent swelling and more than 62 sub-caudal scales were identified as males (Langton, 1989). The phase in the slough cycle was recorded using a three-point scale; freshly sloughed were assigned to Phase 0, dulling ventral scales to Phase 1 and opaque eyes to Phase 2. If a food bolus was detected during handling, this was also recorded.

To reduce the risk of cross-contamination and the potential exposure of *Oo* to clinically healthy snakes, clean handling procedures were used whilst processing snakes and collecting samples. This consisted of wearing nitrile gloves at the field site at all times, changed between the handling of each snake, and sanitising equipment between snakes as well as at the end of each survey day. A biosecurity protocol was established informed by the findings of Rzadkowska *et al*. (2016) (See Supplementary Materials 3.7.1). Dimethyl benzyl ammonium saccharinate (0.1 % active ingredient; Dettol All-in-One Disinfectant spray), was sprayed on any surface that snakes came into contact with and the equipment used, for a minimum contact time of 10 minutes (Rzadkowska *et al*. 2016). This was then rinsed with clean tap water and air dried before the next use.

If skin lesions were detected, then these were swabbed in duplicate using two different MW-100 swabs (Medical Wire & Equipment), moistened with molecular-grade nuclease-free water (Thermo Fisher Scientific). Where snakes had multiple lesions, as many as possible were swabbed with one swab and then again with the second, ensuring consistency by sampling the same lesions. Every fifth snake captured without detectable skin lesions was swabbed continuously along the ventrum from the neck to the vent five times, allowing for a control. A duplicate was taken using the same method. If it was apparent that the same individual had previously been swabbed within the past 24 hours (due to the presence of recognisable features such as scars or trauma), then they were not swabbed until the next capture occasion. Swabs were stored in a portable insulated freezer bag for the duration of the fieldwork and transferred to a dedicated -20°C freezer within 30 minutes of leaving the site.

When skin lesions were detected, their appearance was classified according to their colour and distribution. Skin lesions were also described based on their appearance and were assigned one or more of the following seven characteristics; discolouration, dysecdysis, scale distortion, scale margin erosion, swelling, and ulceration (see Supplementary Materials 3.7.2 for definitions of skin lesion characteristics). The seven characteristics were not mutually exclusive. The distribution of skin lesions was recorded for each of five sections of body length (i.e. the head and then the four quarters along the body thereafter), and whether they occurred on the dorsal, ventral and/or lateral body surfaces (see Fig. 3.1). Photographs of the skin lesions were taken on a digital SLR camera (Pentax K-50 with an 18-55 mm lens).

Captured snakes were also examined for the presence of trauma, with data recorded where the aetiology was strongly suspected to be different to those lesions caused by an infectious disease, such as puncture wounds and lacerations (Supplementary Materials 3.7.2). When swabbing snakes, these areas were avoided in order to reduce the risk of a false positive, due to the likelihood of *Oo* being opportunistic in such scenarios, but not the causative agent of the trauma. A comparison between snakes displaying trauma with concurrent skin lesions and a lack of them is detailed below in section 3.4.5.

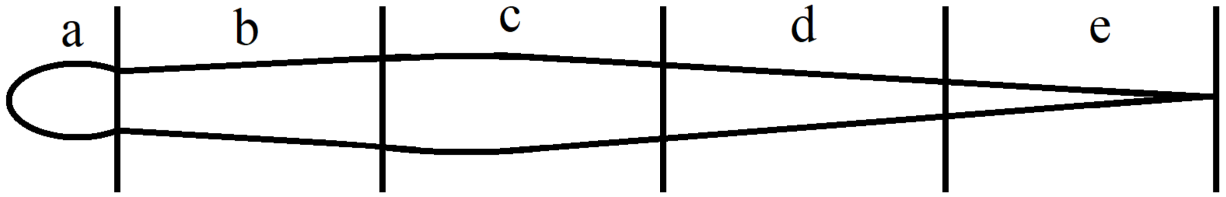


Figure 3.1: A diagram demonstrating how the body length of snakes was divided into the head (a) and four quarters (b-e).

Photographs documenting 33 snakes with skin lesions were randomly selected from a photo library of first season data (i.e. 2019) of 100 cases. Each case was selected using a random number generator (https://www.random.org/) before being reassigned a new number using the same generator. This produced a double-blind sample for the next step; the images were then scored by two observers (SJRA and BL) using our predefined scoring system. This was undertaken to ensure that the terminology used to describe the skin lesions was consistent for both the PhD student and wildlife veterinarian.

A scoring system (4-16) was developed to rank the severity of skin lesions based on their characteristics, number and distribution (See Supplementary Materials 3.7.3). This framework was adapted from Baker *et al*. (2019) to accommodate the variety of skin lesions observed in *N. helvetica* and further categorised skin lesions as very mild (score 4), mild (5-8), moderate (9-12) or severe (13-16). Snakes were also examined for the presence of skin injuries with an appearance consistent with trauma, for example potential predation wounds. When present, details were recorded, and photographs taken as previously described.

3.3.2 Calculating scaled mass index (SMI)

The scaled mass index was used to measure the body condition of captured *N. helvetica* using the following equation from Peig & Green (2009):

$\widehat{M_i} = M_i \left[\frac{L_0}{L_i}\right]^{b_{sma}}$ Where M_i is the body mass, L_0 is an arbitrary value of L (e.g. the arithmetic mean value for the study population), L_i is the linear body measurement of individual i, b_{sma} is the scaling exponent estimated by the standardised major axis (SMA) of M on L, producing $\widehat{M_i}$, the predicted body mass for individual i (Peig & Green, 2009).

For more information, please refer to Chapter 2.

3.3.3 Opportunistic sample collection

Ten grass snake carcasses were collected from the field site (May 2019-September 2021) and examined at a later date at the Institute of Zoology, ZSL London Zoo. Post-mortem examinations (PMEs) were conducted by a ZSL veterinarian using a standardised protocol (Franklinos *et al*., 2017). Briefly, systematic external and internal inspection of all body systems was conducted, supported by microbiological, parasitological and/or histological examination as indicated based on macroscopic findings and as permitted by the state of carcass preservation. Samples of detected skin lesions were stored at -80°C prior to *Oo* rtPCR and fixed in 10% buffered formal saline pending histological examination using routine methods, by an external laboratory. For their methods, please see below.

In addition, snake sloughs encountered in the field were collected and placed in individual paper envelopes before being stored at -20°C. Each slough was then defrosted and visually inspected for the presence of areas of thickening or discolouration, potentially consistent with skin lesions. If these were present, samples were collected using a sterile scalpel blade and stored at -20°C pending rtPCR analysis to determine if *Oo* DNA was present. If more than one lesion was present on a slough, a small number of these were fixed in 10% buffered formal saline for histological examination, as with the tissue samples from carcasses. Sloughs were measured in length (to the nearest cm) and their level of completeness was estimated, allowing for the estimation of the size class of the individual the slough originated from.

3.3.4 Laboratory protocols

3.3.4.1 Real-time PCR

DNA was extracted from swab samples using the protocol described by Franklinos *et al*. (2017). Briefly, 0.035 g of 0.5 mm zirconium microbeads were placed in a 0.5 ml microcentrifuge tube (Boyle *et al.*, 2004). 60 µl of PrepMan Ultra (Applied Biosystems) was added to each sample before being homogenised in a bead beater for 45 seconds. For skin lesion samples collected from sloughs, only 50 µl of PrepMan Ultra was used during the extraction process (Hyatt *et al.*, 2007). All samples were then centrifuged for 30 seconds at 14,500 rpm, before being cycled in the bead beater and centrifuged again. The samples were then heated at 100°C for 10 minutes using a heating block, before being removed and left to cool for 2 minutes at room temperature. Finally, the samples were centrifuged for 3 minutes (14,500 rpm) before the supernatant from each sample was transferred to a different 0.5 ml microcentrifuge tube. In some circumstances the remaining swab tip was inverted and the sample centrifuged another time, allowing for the extraction of the supernatant. The collected supernatant was then diluted to a 1 in 10 solution, using PCR-grade water.

The same process was completed using new sterile MW-100 swabs, which were later used as negative extraction controls, to provide a check that no cross-contamination of samples occurred during the extraction process.

The detection of *Oo* was completed on DNA extracts from both skin swabs and skin lesions using the standard real-time PCR (rtPCR) protocol following Bohuski *et al.* (2015), running the plates for 60 cycles using a StepOnePlus™ Real-Time PCR Machine

(ThermoFisher Scientific). *Ophidiomyces ophidiicola* was targeted using the primers Oo-rt-ITS-F and Oo-rt-ITS-R, using 5 µl of template DNA in a 20 µl reaction.

The cycle threshold (Ct), defined as the number of cycles needed for the signal from the fluorescent dye (MGB) to exceed that of background level (Denman & Sweeney, 2005), was recorded for each result. The rtPCR was run in duplicate per DNA extract and any ambiguous results were repeated a maximum of three times, until the amplification across both wells were consistent. If this was not possible, they were labelled as 'undetermined'. For the purpose of this study, barred grass snakes were considered positive if one or both of the duplicate skin swabs tested real-time PCR positive for *Oo* DNA, within 36 cycles (Bohuski *et al*., 2015).

3.3.4.2 Internal control

An internal control PCR was used, targeting a section of the host's Early B-cell Factor 3 (EBF3) gene (an ultra-conserved non-coding element) to determine if inhibition had occurred during rtPCR (Leung *et al*., 2017). This would permit detection of false negatives for *Oo* DNA using the rtPCR, by providing evidence for the absence of host DNA amplification. The primers for this target gene have previously been shown to amplify in the presence of a wide range of amphibian and reptile species' DNA, including the viperine snake (*Natrix maura*; Leung *et al*., 2017). Protocol optimisation was practised exploring the outcome of altering the annealing temperature, number of cycles and volume of DNA template. 10 µl reactions were run containing 2 µl 5x HOT FIREPol® (Soils Biodyne) Master Mix and 0.6 µM of both EBF3N_F and EBF3N_R primers. 2 µl of the diluted DNA extract was added to each of the wells, with 2 µl also used for both the positive and negative controls.

Once the plate had been sealed and centrifuged, it was then placed in a GeneAmp® PCR System 9700 (Applied Biosystems) for 35 cycles under the following conditions: A 15 minute hold at the beginning at 95°C, then 35 cycles of denaturation at 95°C for 20 seconds, annealing at 60°C for 1 minute, extension at 72°C for one minute, followed by a final hold at 72°C for 10 minutes.

For the gel electrophoresis, 2 µl loading dye (containing 300 µl 5x loading dye, 300 µl of PCR water and 1.2 µl of GelRed (Biotium)) was added to 5 µl of the PCR products from each sample, before being loaded into a 1-2% agarose gel. 2 µl of HyperLadder™ 50 bp (Bioline) was loaded into the first well in each row, to act as the weight marker. The gel was left to run for 30 minutes, with a power supply of 100 V, 300 mA, and 100 W. Once the gel had been run, it was then visualised using ultraviolet light (G:BOX F3, Syngene).

3.3.4.5 Histological staining

Two staining methods were used in the histopathological investigations to detect the presence of fungal hyphae and arthroconidia consistent with *Oo* and lesions associated with ophidiomycosis within the samples of skin lesions from PME and skin sloughs. Haematoxylin and eosin (H&E) stains were used as routine for histopathological examination. Periodic acid-Schiff (PAS) stain aids in the identification of polysaccharides and therefore enables enhanced visualisation of *Oo*, since the cell walls of fungi contain high levels of carbohydrate. Gram staining was used to determine the potential presence of Gram-positive and Gram-negative bacteria within the histopathological samples. All histopathological examinations were conducted by a board-certified veterinary pathologist at IDEXX laboratories.

3.3.5 Statistical analysis

Generalised Linear Mixed Models (GLMMs) were used to determine which of the following fixed factors: sex, snout-to-vent length, slough cycle, presence of trauma, and month of capture were most closely associated with the occurrence of skin lesions in *N. helvetica*. As a small number of recaptured snakes were included in the analysis that were recaptured between years, individual snake was included as a random factor. Size class and the scaled mass index of individuals were not included in the models since these results were evidently correlated with snout-to-vent length. The generalized variance-inflation factor (GVIF) was used to test for evidence of correlation between the remaining factors (Fox & Monette, 1992). Analyses were conducted using R (version 4.2.2), using the package lme4 (Bates *et al.*, 2015; R Core Team, 2022).

3.4 Results

The annual prevalence of skin lesions varied significantly across the study period, with 26.1% (77/295) of snakes encountered in 2019 with skin lesions, 14.5% (35/241) in 2020, and 36.9% (79/214) in 2021, $\chi^2 = 29.79$, df = 2, p = <0.01. A total of 191 individuals with skin lesions were captured over the study period. The results detailed in sections 3.4.1 to 3.4.7 include data for the first encounter of each individual snake only, in order to avoid pseudoreplication within the analyses. Section 3.4.8 provides information on recapture and serial monitoring of snakes. Finally, section 3.4.9 utilises data from the first capture of each individual each year, including the small number of recaptures of some snake across years. Across the three years, there were 42 captures of snakes found basking in the open, rather than found under ACOs: 18 (42.9%) of these had detectable skin lesions, which is a significantly greater percentage than for snakes caught under ACOs, $\chi^2 = 7.09$, df = 1, p = <0.01. However, the majority of these (n = 17) occurred in 2021.

3.4.1 Character and distribution of skin lesions among snakes

3.4.1.1 Skin lesion characteristics

Fleiss' Kappa was used to judge the interrater agreement between the two observers, which showed very high levels of agreement across the samples (K = 0.936, p = 1; Table 3.1). Given the high consistency between the two observers, SJRA subsequently reviewed the skin lesion images and coded their characteristics.

Table 3.1: The percentage agreement between two observers (PhD student and wildlife veterinarian) regarding the skin lesion characteristics of thirty-three barred grass snakes (*Natrix helvetica*), scored for the seven different categories.

	Crusting	Discolouration	Dysecdysis	Scale distortion	Scale margin erosion	Swelling	Ulceration
Agreement (%)	100	100	100	96.97	96.97	93.939	100

Examples of the most frequently encountered skin lesion characteristics in grass snakes are shown in Fig. 3.2. The most common skin lesion characteristic was discolouration seen in 97.9% of cases (187/191): lesions were tan, red, brown, or a combination thereof (Fig. 3.3). Discolouration was typically concurrent with crusting (89.5%; 171/191) and scale margin erosion (73.2%; 140/191). Less frequently encountered skin lesion characteristics were swelling (26.7%; 51/191) and distortion of scales (10.9%; 21/191; Fig. 3.4), whilst the rarest characteristics recorded were ulceration (7.7%; 13/191), and dysecdysis (2.9%; 5/191).

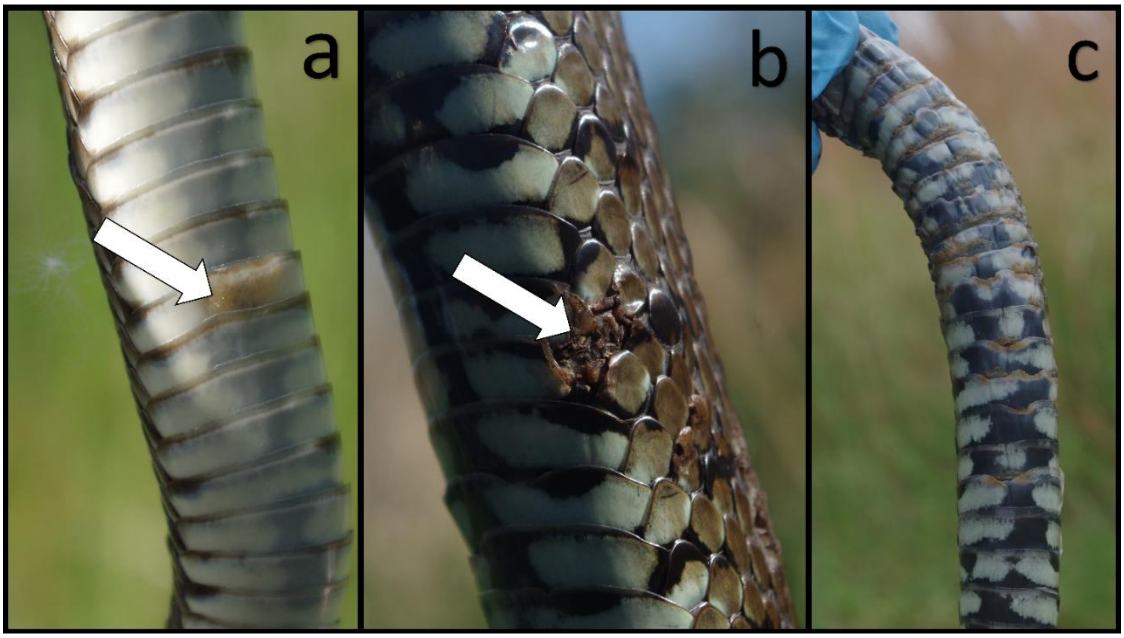


Figure 3.2: Examples of the most commonly encountered skin lesion characteristics observed in barred grass snakes (*Natrix helvetica*). These include discolouration (a), discolouration, crusting, and ulceration (b), and discolouration, crusting, and scale margin erosion (c). The severity score of lesions in (a-c) was recorded as mild, moderate, and severe respectively.

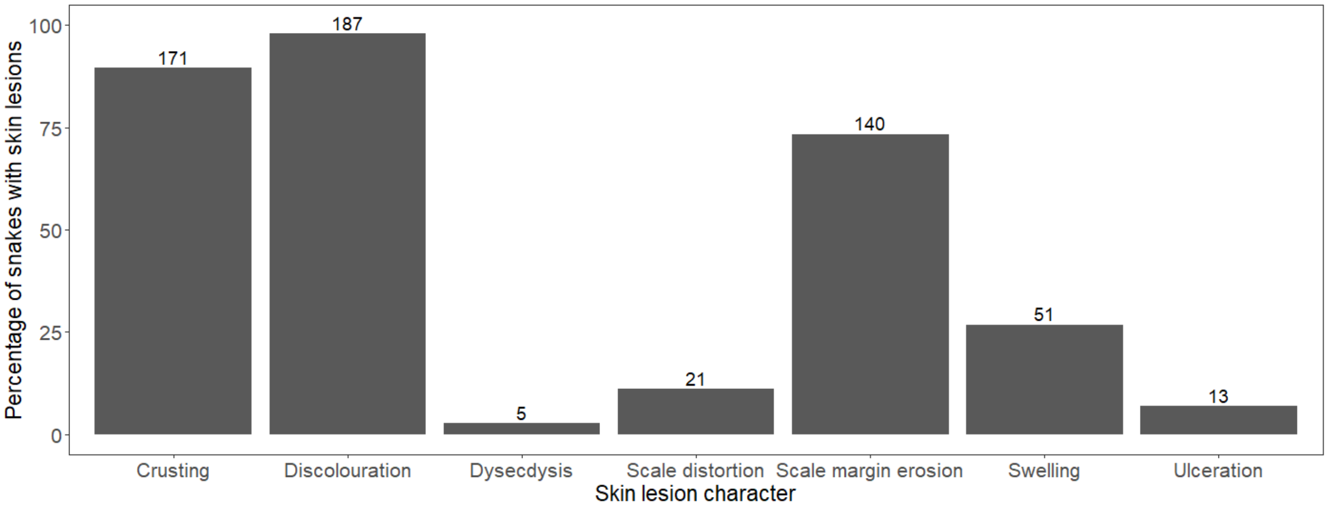


Figure 3.3: The percentage of barred grass snakes (*Natrix helvetica*) with detected skin lesions that were categorised according to the following non-mutually exclusive characteristics: crusting, discolouration, dysecdysis, scale distortion, scale margin erosion, swelling and ulceration. The number of snakes with each skin lesion characteristic is presented on each bar. A total of 191 snakes with skin lesions were caught during the three year period 2019-2021, at the study site in Norfolk, England.

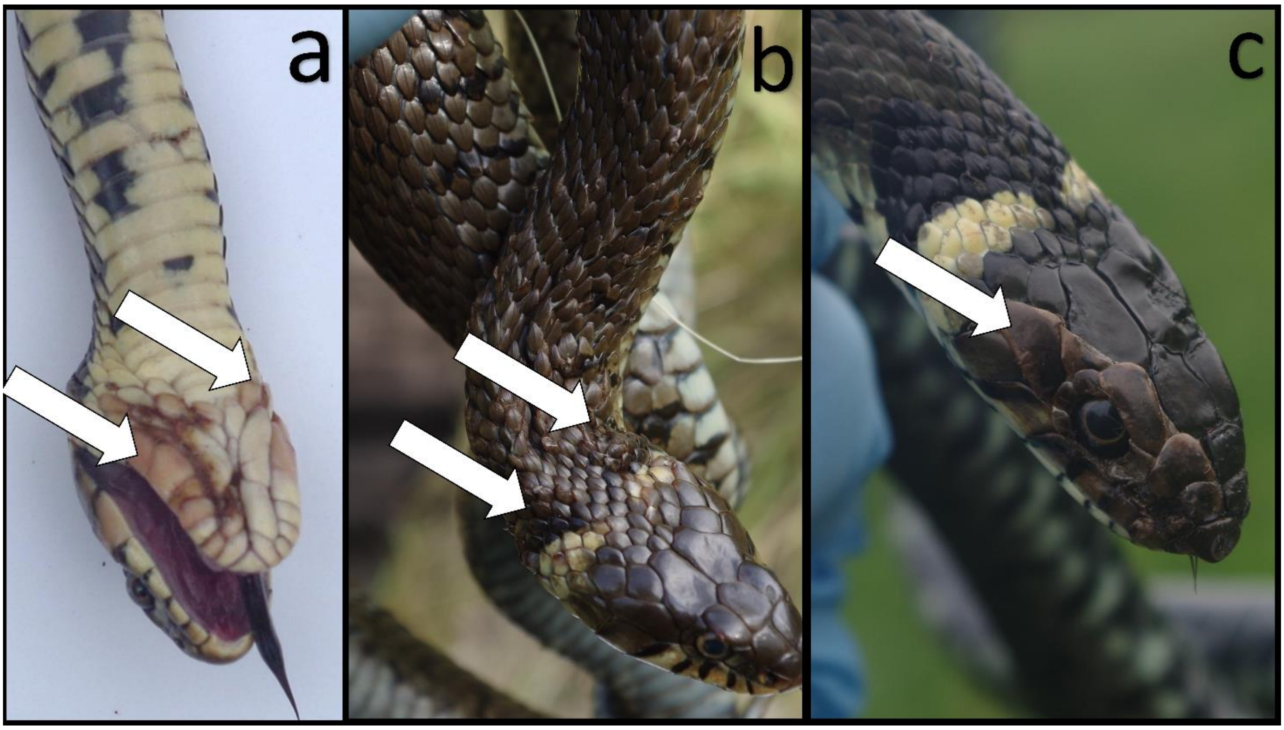


Figure 3.4: Examples of the more rarely encountered skin lesion characteristics observed in barred grass snakes (*Natrix helvetica*). These include swelling (a), distorted scales (b), and dysecdysis (c).

3.4.1.2 Skin lesion distribution

The distribution of skin lesions varied along the body length and body surface of snakes (Fig. 3.5a). Snakes most commonly had skin lesions on the mid-body section, affecting 50.7% (97/191) on the 1st, 56.5% on the 2nd and 53.9% on the 3rd quarter, as compared with other regions of the body. Only 34.5% of snakes with skin lesions involved the head and 21.9% the 4th quarter 21.9%. This demonstrates that skin lesion occurrence was fairly even along the body of snakes, with fewer occurrences on the head and tail (i.e. 4th quarter). More than two thirds (71.7%) of snakes had skin lesions present in more than one region.

The ventral surface had the highest frequency of observed skin lesions with 96.9% (185/191) of snakes captured having skin lesions present there (Fig. 3.5b). Only 8.4% (16/191) of snakes with lesions occurred on their lateral surfaces, and 6.8% (13/191) on the dorsal surface.

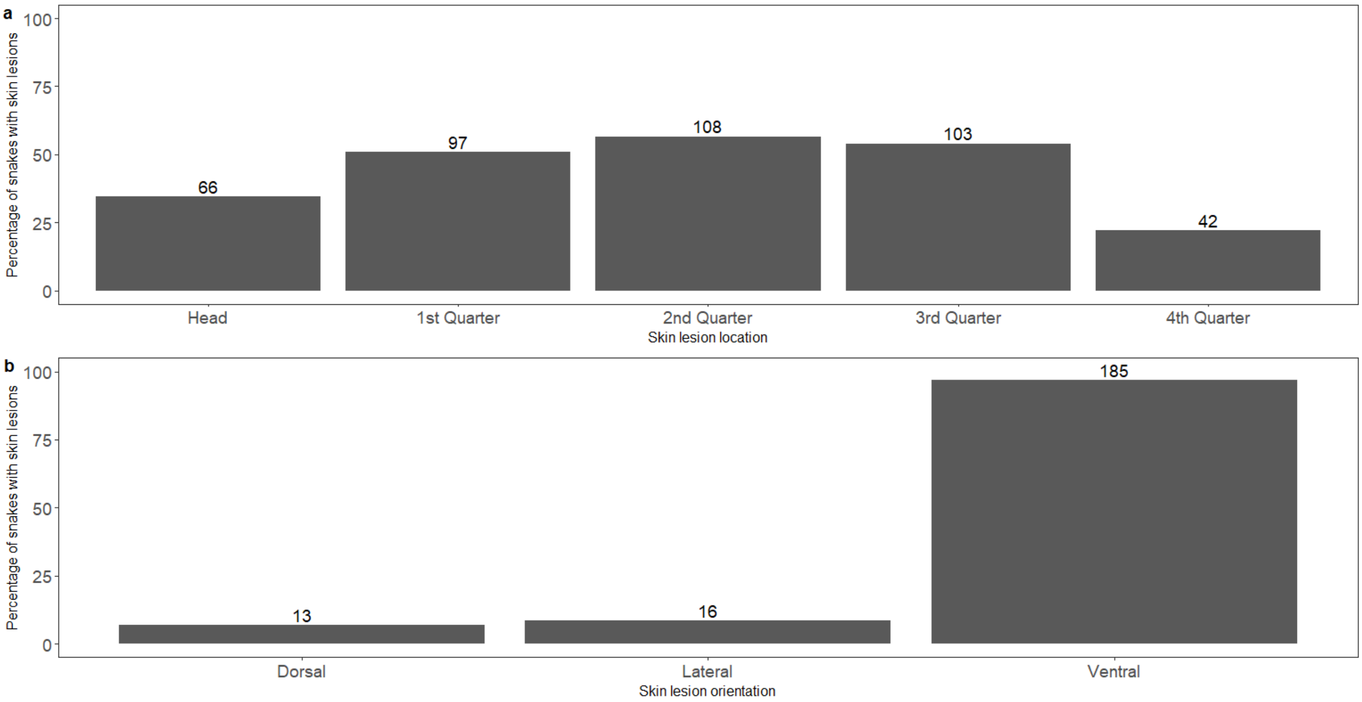


Figure 3.5: The percentage of barred grass snakes (*Natrix helvetica*) with detected skin lesions categorised by location along the body length (a) and body surface (b). The number of snakes with skin lesions observed in each location are presented on the bars. A total of 191 snakes with skin lesions were caught during the period 2019-2021, at the study site in Norfolk, England.

3.4.1.3 Skin lesion severity

The most common category of skin lesion severity was mild (51.3%; 98/191), closely followed by moderate (34.0%; Fig. 3.6a), with very few capture occasions classified as very mild (5.8%) or severe (8.9%). This is reflected in the skin lesion severity scores (Fig. 3.6b), which do not follow a normal distribution, (Shapiro-Wilk, W(194) = 0.96, p = <0.01).

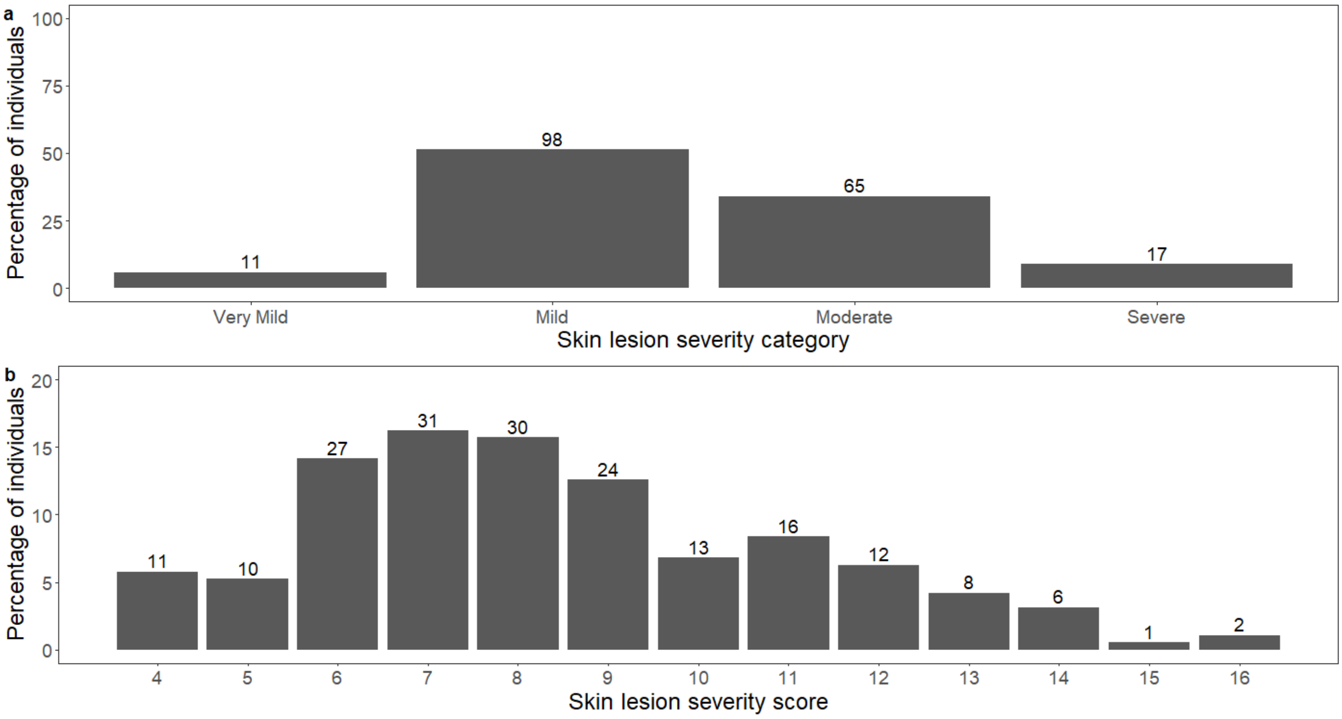


Figure 3.6: The percentage of barred grass snakes (*Natrix helvetica*) in each of the skin lesion severity categories (a) and skin lesion severity scores (b), (n = 191). Note that the y-axis in (b) has been reduced to 0-20% so that the bars are legible. Numbers over bars indicate the frequency of individuals in each category.

3.4.2 Opportunistic examination of snake carcasses and skin slough specimens

3.4.2.1 Post-mortem examinations

Post-mortem examinations were conducted on one neonate and three adult barred grass snakes, with skin lesions present were found dead over the period 2019-2021. One of the adults was female, the other carcasses were of indeterminate sex due to their size and state of preservation. A sample of skin lesion (Fig. 3.7) from the adult female was rtPCR-positive for *Oo*, and fixed samples were subsequently subjected to histopathological examination to further investigate their aetiology (Fig 3.9). Skin lesions from the neonate (with mild skin lesions on the underside of the head) and the two other snakes tested rtPCR-negative for *Oo* DNA. The proximate cause of death of the adult female snake and one of the other adults was undetermined, due to a poor state of carcass preservation. The cause of death for the remaining two snakes was determined to be trauma. No skin lesions were detected on the remaining six grass snakes examined post mortem from the study site, and skin swabs collected from these animals tested rtPCR-negative or inconclusive (amplification of samples was not consistent), for *Oo* DNA.

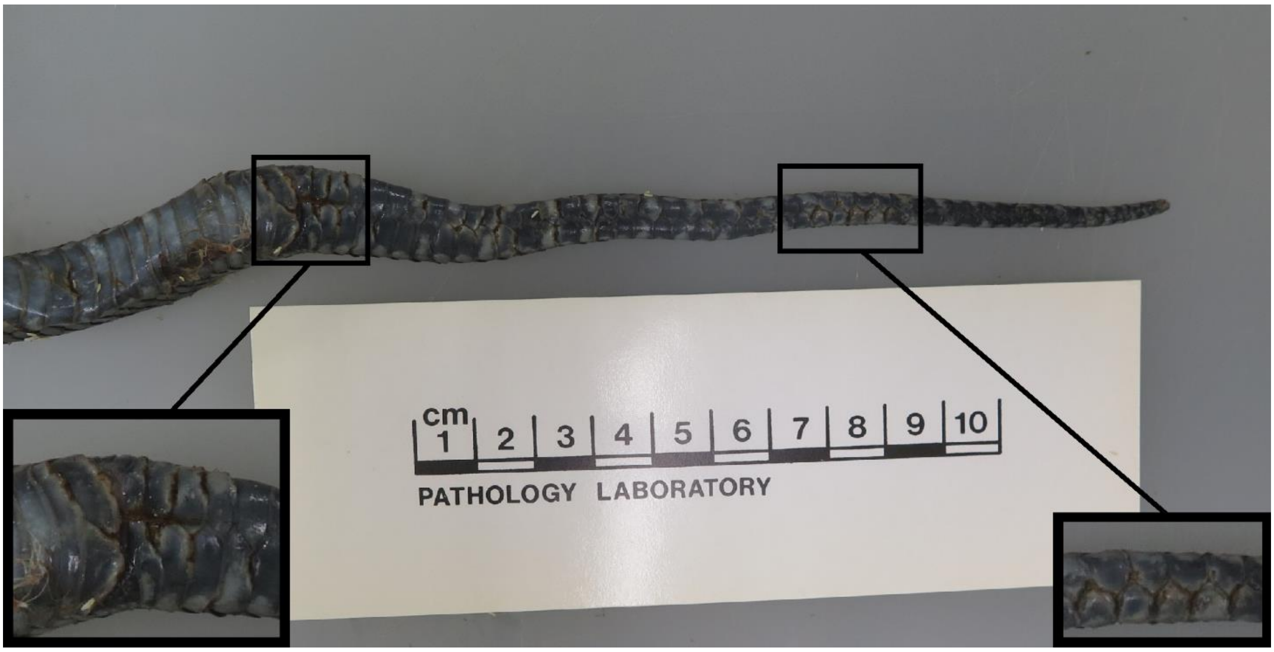


Figure 3.7: Skin lesions along the tail section of the adult female barred grass snake (*Natrix helvetica*) that tested real-time PCR-positive for *Ophidiomyces ophidiicola* DNA, with additional histopathological confirmation of ophidiomycosis (See Fig 3.9). Severe skin lesions characterised as discolouration, crusting and scale margin erosion are present; skin lesions around the cloaca and further along the tail are shown as inset, at higher magnification.

3.4.2.2 Skin slough examinations

A total of 42 skin sloughs were collected from the environment across the study period 2019-2021: 39.0% (n = 16) had evidence of lesions (Fig. 3.3), of which 56.3% (9/16) tested rtPCR-positive (Table 3.2). However, no apparent difference was noted in the appearance of skin slough lesions in *Oo* rtPCR-positive versus negative samples.

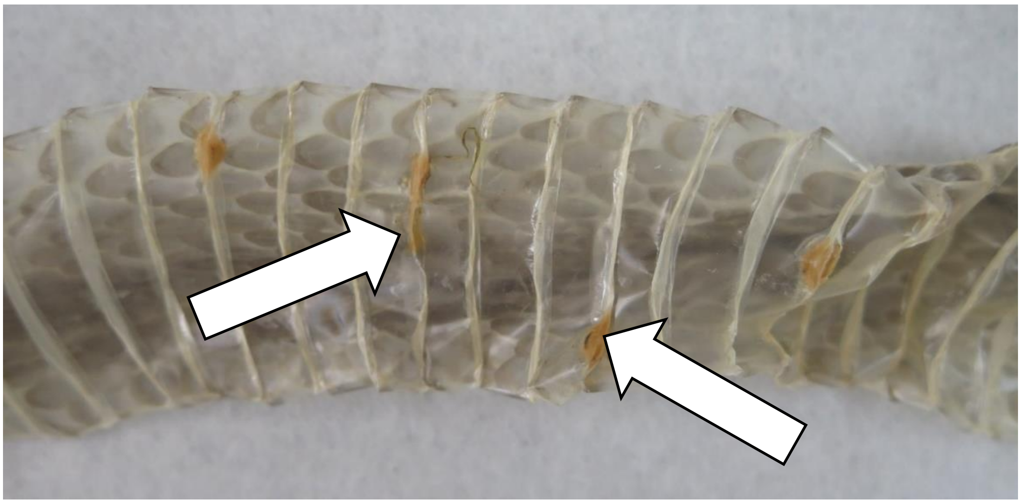


Figure 3.8: Barred grass snake (*Natrix helvetica*) skin slough with multiple tan-coloured lesions (indicated by arrows) which tested real-time PCR-positive for *Ophidiomyces ophidiicola* DNA.

Table 3.2: Number of barred grass snake (*Natrix helvetica*) sloughs collected, compared with how many of these had lesions present and were subsequently real-time PCR-positive for *Ophidiomyces ophidiicola*. The years marked with an asterisk (*) indicate samples collected by previous MSc students, prior to this PhD study period. Seven of the samples collected in 2016 were included in the study by Ladner *et al*. (2022), where *Oo* identification was confirmed by culture and sequencing.

Survey year	Sloughs collected	Sloughs with lesions detected (%)	Slough lesions that tested rtPCR positive (%)
2016*	34	32.4 (n = 11)	90.9 (n = 10)
2017*	23	65.2 (n = 15)	66.6 (n = 10)
2018	0	NA	NA
2019	15	26.7 (n = 4)	75.0 (n = 3)
2020	14	28.6 (n = 4)	75.0 (n = 3)
2021	13	61.5 (n = 8)	37.5 (n = 3)

Of the skin sloughs collected over the period 2019-2021 inclusive, for which size data were available, rtPCR-positive lesions were tested from a total of 6 sub-adult and 3 adult snakes. Samples of apparently normal skin sloughs from 10 snakes (of all size classes) as a control all tested rtPCR-negative for *Oo*. Negative or inconclusive samples (especially those with lesions) underwent repeat rtPCR in order to provide confidence in the results.

3.4.2.3 Histopathological examination

Histopathological examination of single lesions from five sloughs using H&E and PAS staining revealed the presence of fungal hyphae (n=4) and arthroconidia (n=3) with a morphology consistent with *Oo* (Figs. 3.9 & 3.10). Three skin lesion samples from the adult female described above in 3.4.2.1 each had fungal hyphae and arthroconidia present. Four of the slough samples with fungal hyphae and/or arthroconidia were rtPCR-positive for *Oo* DNA, with different lesions being tested for both the histopathology and rtPCR. Across multiple samples within the affected areas, there was an accumulation of eosinophilic acellular material which was interpreted as keratin, proteinaceous, and/or necrotic material. There were a large number of PAS positive fungal hyphae observed, septated, and branching at 1-3 µm in diameter, whilst the arthroconidia were rectangular in shape, approximately 2 µm x 4 µm in size forming dense aggregates. A mix of Gram-negative and Gram-positive bacteria were also present in multiple skin and slough lesions, perhaps indicating a secondary infection.

Three apparently normal slough samples with no histological evidence of ophidiomycosis tested rtPCR-negative for *Oo* DNA.

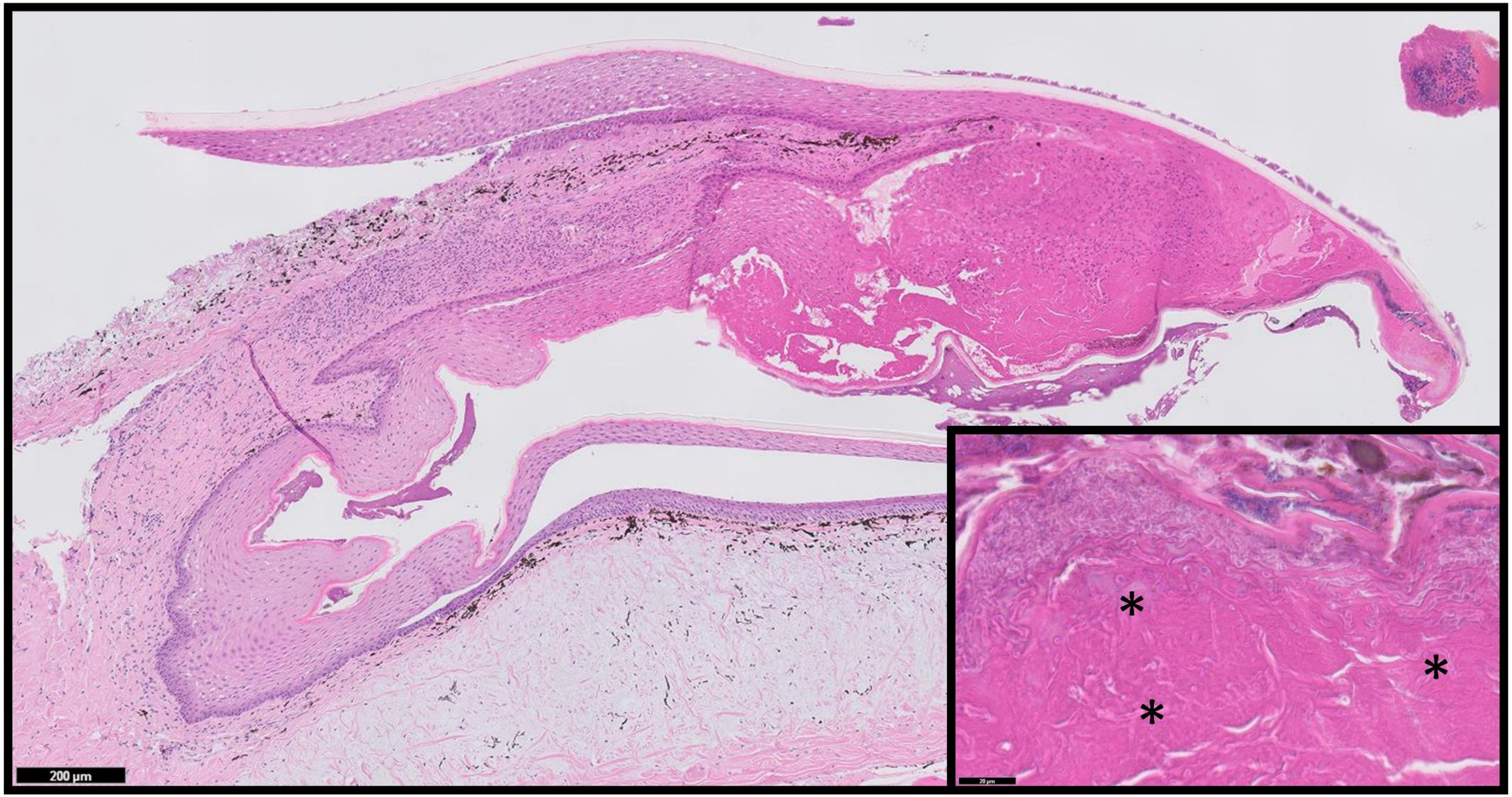


Figure 3.9 Photomicrograph of skin lesions from an adult female barred grass snake (*Natrix helvetica*) revealed areas of epidermal thickening and necrosis, with abundant superficial fungal hyphal elements (asterisks) associated with areas of dermatitis. H&E stain, 5x magnification and 20x for inset.

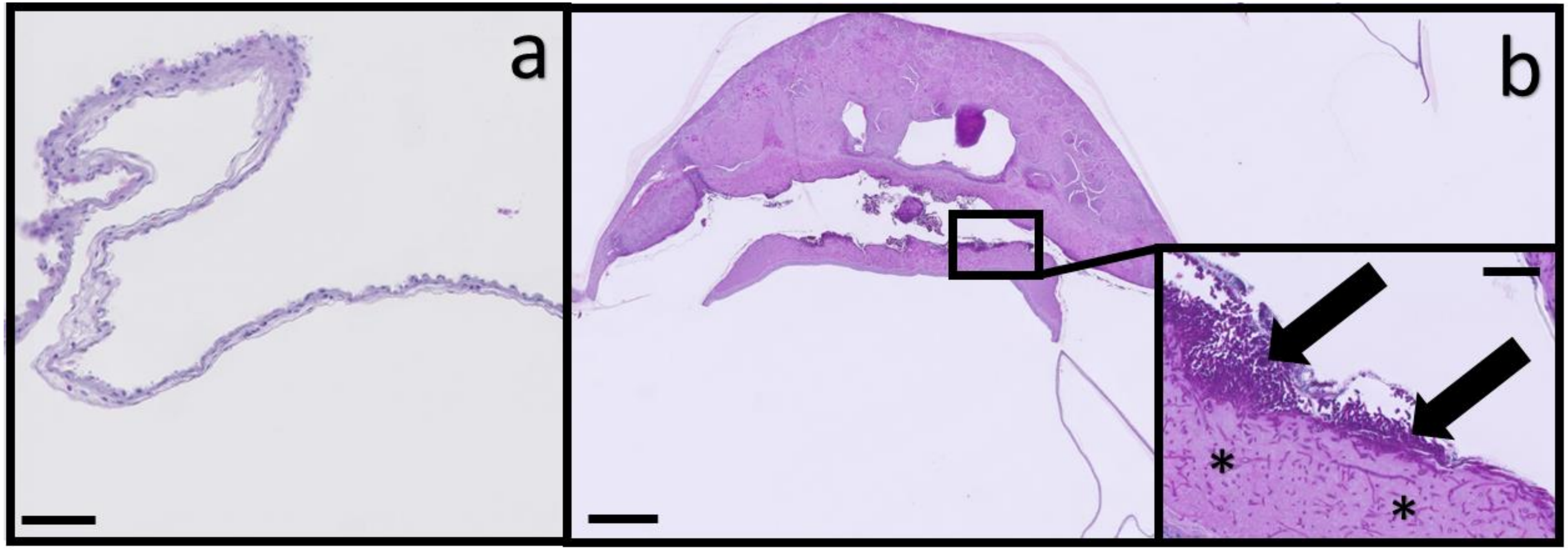


Figure 3.10: Photomicrograph of sloughed skin samples from barred grass snakes (*Natrix helvetica*) (a) is an example of apparently normal sloughed skin which shows the typical thin layer structure, whereas (b) is an example of a lesion from a sloughed skin which revealed areas of epidermal thickening and necrosis, with abundant superficial fungal hyphal elements (asterisks) and arthroconidia (arrows) consistent with *Ophidiomyces ophidiicola*. PAS stain, 10x magnification (a), 2x (b), and 20x (inset).

3.4.2.4 Real-time PCR results from skin swabs collected from live snakes

Over the 3-year study period, the majority of skin swabs collected from 271 live snake captures with skin lesions tested rtPCR-positive for *Oo* (i.e. 78.7%; 215/271) of the first duplicate, and 69.3% (188/271) of the second duplicate, with no significant difference between the set results (paired t-test, $t = 0.37$, $df = 4$, $p = 0.73$). In contrast, a minority of skin swabs from snakes without detected skin lesions tested rtPCR-positive (i.e. 2.5% (4/165) of the first duplicate and 3.0% (5/165) of the second duplicate), with no significant difference between the set results (paired t-test, $t = 0.71$, $df = 4$, $p = 0.52$). In summary, these findings support the hypothesis that the majority of the skin lesions observed in *N. helvetica* were caused by ophidiomycosis (Table 3.3).

Table 3.3: Results of real-time PCR for detection of *Ophidiomyces ophidiicola* DNA in skin swabs from barred grass snakes (*Natrix helvetica*), 2019-2021 inclusive. For the purpose of this study, barred grass snakes were considered positive if one or both of the duplicate skin swabs tested real-time PCR positive for *Oo* DNA.

Year	**With skin lesions**		**Without skin lesions**	
2019	Positive	86 (86.0%)	Positive	2 (3.3%)
	Negative	14 (14.0%)	Negative	59 (96.7%)
2020	Positive	41 (82.0%)	Positive	3 (5.1%)
	Negative	9 (18.0%)	Negative	56 (94.1%)
2021	Positive	105 (86.8%)	Positive	3 (6.3%)
	Negative	16 (13.2%)	Negative	45 (93.7%)

The mean cycle threshold value of the rtPCR was compared across the skin lesion severity score groupings. There was a significant difference between the Ct values for very mild, mild, moderate, and severe skin lesions (One-way ANOVA, $F(3, 413) = 14.37$, $p = < 0.01$). Snakes with skin lesions in the greater severity score categories had lower Ct values on average than milder cases (Fig 3.11). This finding is consistent with greater *Oo* load in samples collected from snakes with more severe lesions since Ct values are proportional to the amount of target DNA within a sample, with lower Ct values indicating that more DNA is present.

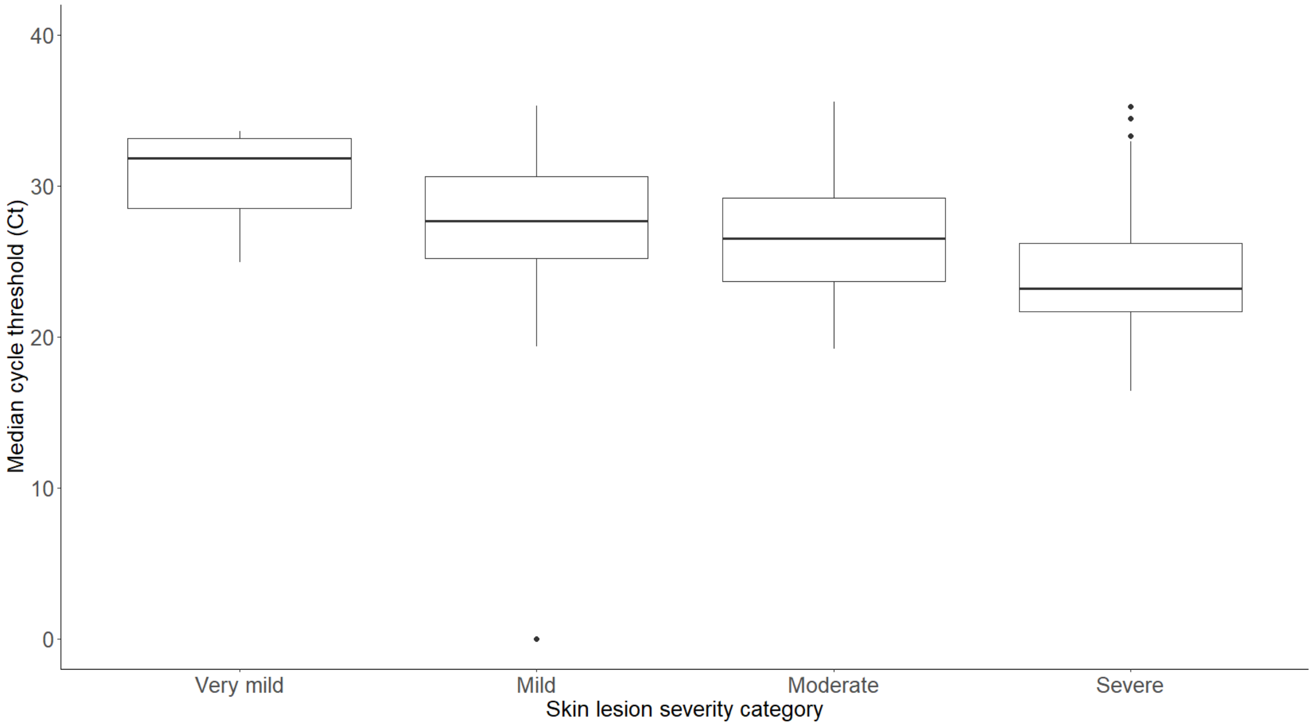


Figure 3.11: Cycle threshold (Ct) value from each real-time PCR positive sample from both swab duplicates, within the four skin lesion severity categories. Results from 414 rtPCR tests are included, corresponding to snakes in each of the four skin lesion severity categories listed. Plotted are the medians, interquartile ranges (indicated by the boxes), ranges (lines), and outliers (black dots).

3.4.2.5 Internal control

The internal control PCR targeting the EBF3N gene was run on DNA extracts from 469 skin swabs which tested rtPCR-negative for *Oo*, from which amplification was detected in 97.2% (226/232) from the first duplicate swab, and 100% (237/237) of swabs from the second duplicate (Fig. 3.12). The 6 skin swabs that tested internal control PCR negative were excluded from the study, since the results indicate that either DNA quality/quantity was insufficient for rtPCR testing, or that PCR inhibition may have occurred.

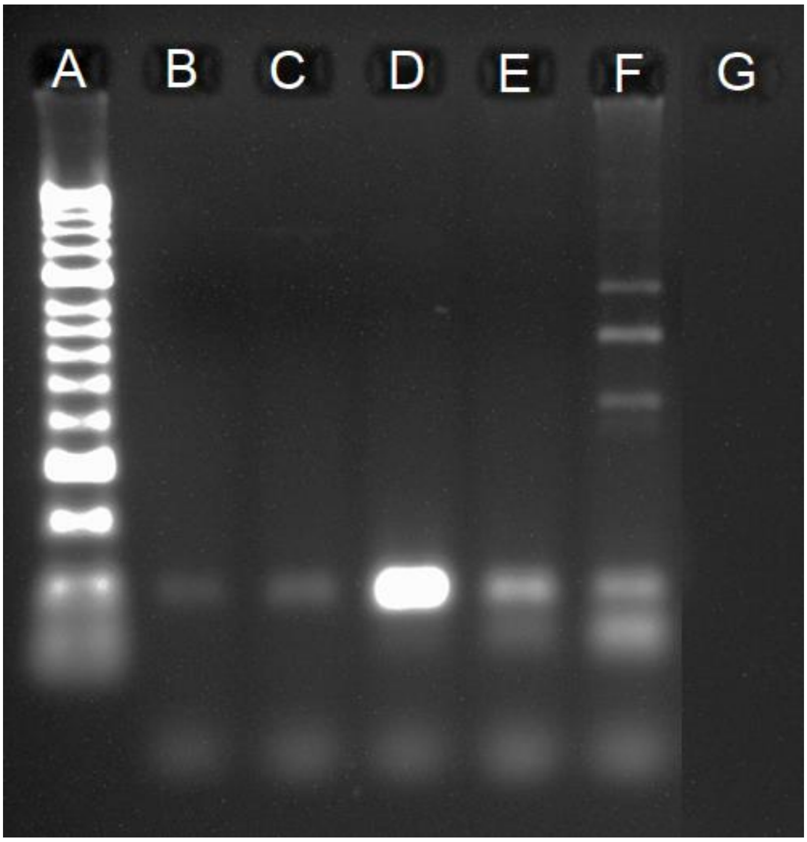


Figure 3.12: Part of an internal control PCR gel showing the DNA ladder (A), four positive samples (B-E), positive control (F), and negative control (G). Despite their variability in fluorescence, all of the samples taken from barred grass snakes (*Natrix helvetica*) show amplification products consistent in size with the EBF3N gene, approximately 80 base pairs in length.

3.4.3 Seasonality of skin lesions

The occurrence of skin lesions within the population of *N. helvetica* peaked in May during 2019 (44.1%, 34/77), July in 2020 (88.6%, 31/35), and June in 2021 (37.9%, 30/79). The 2020 field season was truncated due to impacts of the COVID-19 pandemic, which meant that surveys could not be conducted before July. Because of the missed months of May and June 2020, it is not known if the peak seasonality of skin lesions was skewed for that year, but based on data from 2019 and 2020, it is likely (Table 3.4).

Across all three years, a decreasing number of snakes with skin lesions were detected after July, as the active season progressed towards winter, and snakes started to disperse from the site. Excluding 2020 from the analysis, there was a significant difference between the month of capture in the frequency of snakes caught with skin lesions, $\chi^2 = 25.73$, df = 4, p = <0.01, demonstrating a seasonal link between skin lesion occurrence and the month of capture.

Table 3.4: The frequency of captures of the barred grass snake (*Natrix helvetica*) with skin lesions, during each month of the active field season across the three years that data were collected, compared to the number of captures each month. The percentage of total captures of snakes with skin lesions each month is provided in brackets.

	2019	2020	2021
May	34/101 (33.7%)	NA	9/29 (31.0%)
June	23/110 (20.9%)	NA	30/102 (29.4%)
July	13/101 (12.9%)	31/262 (11.8%)	15/131 (11.5%)
August	5/92 (5.4%)	3/62 (4.8%)	17/71 (23.9%)
September	2/33 (6.1%)	1/33 (3.0%)	8/37 (21.6%)

3.4.4 Size class and sex of snakes with skin lesions

3.4.4.1 Size class

The size class of individuals was based on their snout-to-vent length (SVL) with neonates regarded as snakes with an SVL below 20 cm, sub-adults with an SVL between 20-50 cm, and adults with an SVL greater than 50 cm. For more information on the rationale behind this, see Chapter 2. The modal size class of captured *N. helvetica* was sub-adult (54.6%, 384/791), followed by adult (42.1%), and neonate (3.3%). However, the majority of snakes with skin lesions were adult (72.8%, 139/191; Fig. 3.13). Sub-adults comprised 25.1% of snakes with skin lesions, and neonates only 2.1% of these captures. There was a significant association between the presence of skin lesions and size class, $\chi^2 = 97.45$, df = 2, p = <0.01.

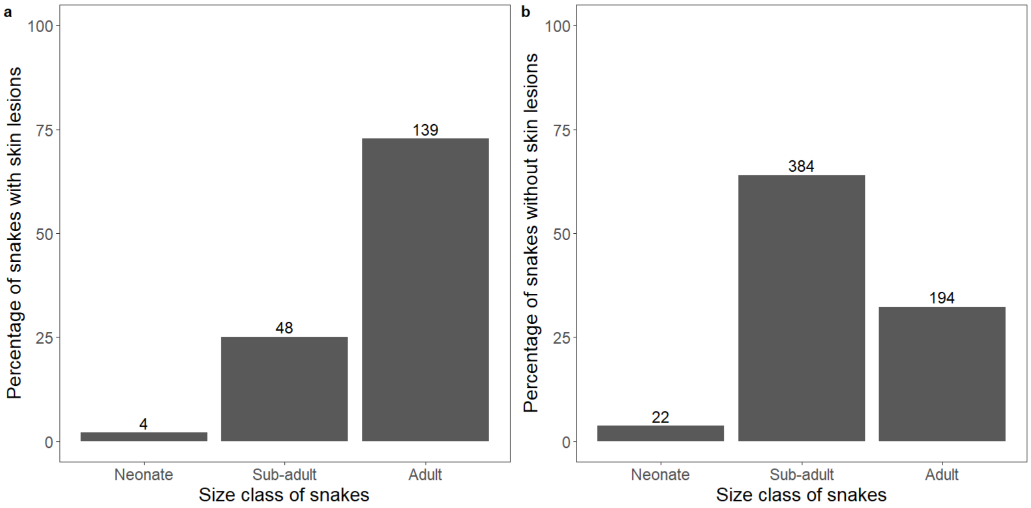


Figure 3.13: A comparison between the percentages of barred grass snakes (*Natrix helvetica*) captured 2019-2021 inclusive, belonging to each size class. Those snakes with skin lesions (n = 191) are on the left (a), those without (n = 600) on the right (b). The number of snakes in each category is indicated above the bars.

3.4.4.2 *Sex*

The number of male and female snakes without skin lesions was almost identical, with 294 females (49.0%), and 306 males (51.0%) captured during the sampling period (Fig. 3.14). There was a slight male bias in snakes with skin lesions, with them representing 54.9% (105/191) of captures, but this was not statistically significant, $\chi^2 = 0.50$, df = 1, p = 0.48.

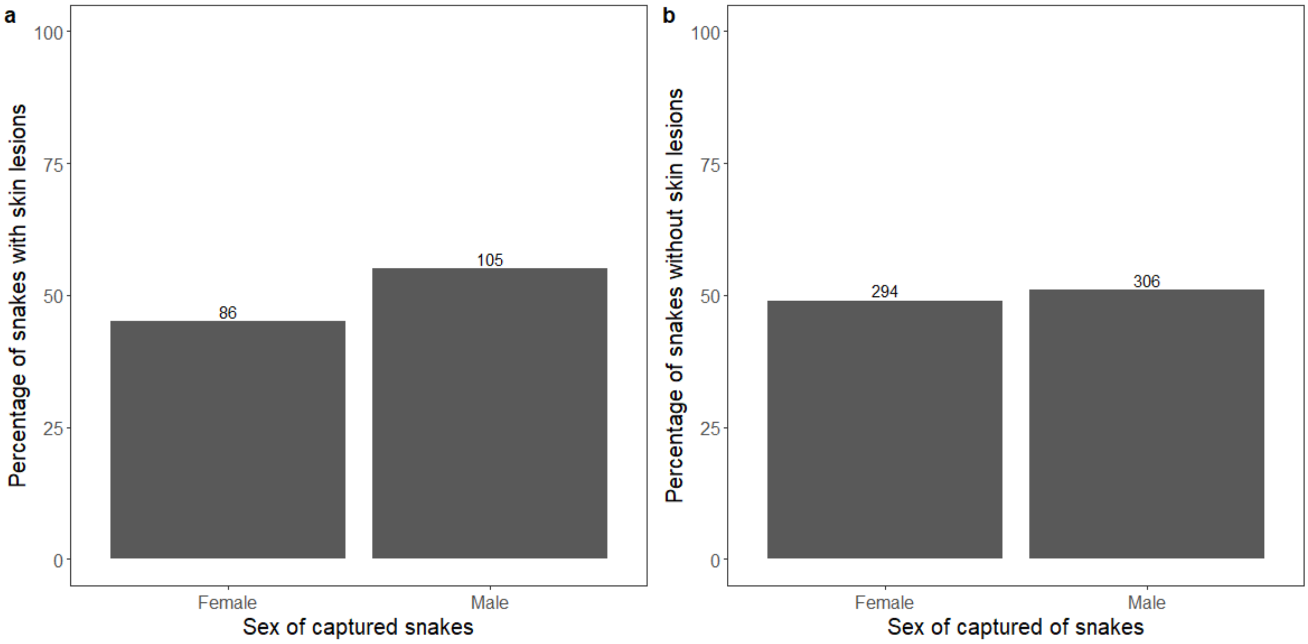


Figure 3.14: A comparison between the percentages of barred grass snakes (*Natrix helvetica*) captured 2019-2021 inclusive, belonging to each sex. Those snakes with skin lesions (n = 191) are on the left (a), those without (n = 600) on the right (b). The number of snakes in each category is indicated above the bars.

3.4.4.3 Slough cycle

The majority of snakes with skin lesions were observed to be in Phase 0 of the slough cycle (46.9%, 83/177; Fig. 3.15a), followed by Phase 1 (33.3%), and Phase 2 (19.8%). A similar pattern was seen in snakes without skin lesions (Fig. 3.15b), with a larger majority of captured individuals in Phase 0 (71%, 419/590). There was a significant association between slough cycle and the presence of skin lesions, $\chi^2 = 35.98$, df = 2, p = <0.01. A total of 24 individuals from both groups of snakes were removed from the analysis, as they were captured during the pilot stage of the project, before individuals were assigned to the three stages of the slough cycle.

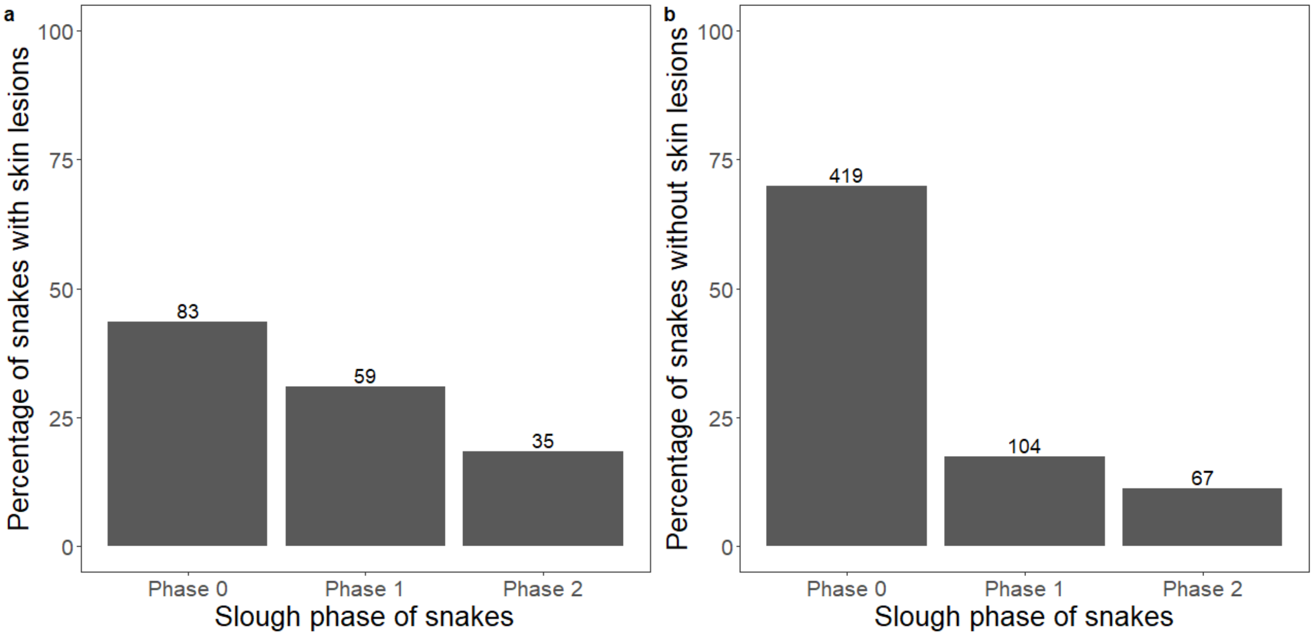


Figure 3.15: The percentage of barred grass snakes (*Natrix helvetica*) captured with skin lesions 2019-2021 inclusive, recorded in each of the three slough phases (a), and those without skin lesions (b). The number of snakes within each category are presented on the bars. Please note that both parts a and b represent different groups of snakes.

3.4.5 Trauma

Skin injuries consistent with trauma were observed in 26 snakes with concurrent skin lesions over the study period, of which the most commonly recorded were damage to the tail (n = 19), lacerations (n = 3), puncture wounds (n = 3), and combinations of the above (n = 1). An additional 20 snakes without skin lesions had injuries consistent with trauma. The type of these traumatic signs was similar to those seen in snakes with skin lesions; damage to the tail (n = 15), lacerations (n = 2), puncture wounds (n = 2), and combinations of the above (n = 1).

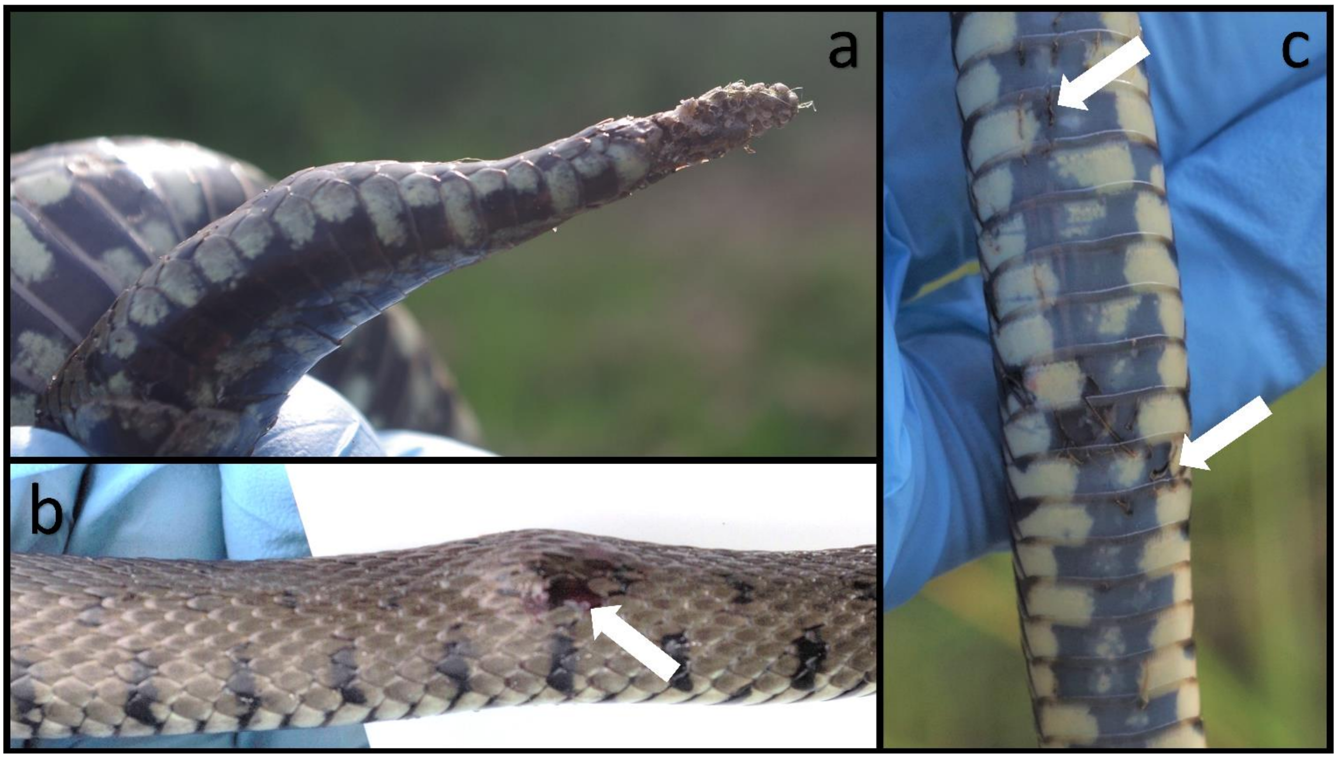


Figure 3.16: Examples of skin injury consistent with trauma observed in the barred grass snake (*Natrix helvetica*), the end of the tail missing with localised necrosis (a), puncture wound with bleeding and localised swelling consistent with inflammation (b), and linear lacerations to the underside of a snake (c).

The percentage of individual snake captures with observed skin injuries consistent with trauma was significantly higher in those with concurrent skin lesions than without, $\chi^2 = 27.95$, df = 1, p = <0.01. For snakes with skin lesions, 13.6% (26/191) of individuals were observed to have skin injury consistent with trauma, as compared with only 3.3% (20/600) of snakes without skin lesions (Fig. 3.17). This remains true when using all the available data, including repeat captures, and not just those from the initial capture, with snakes with skin lesions (16.6%; 45/271) having a stronger association with trauma, than those without skin lesions (3.9%; 35/884), $\chi^2 = 42.2$, df = 1, p = <0.01.

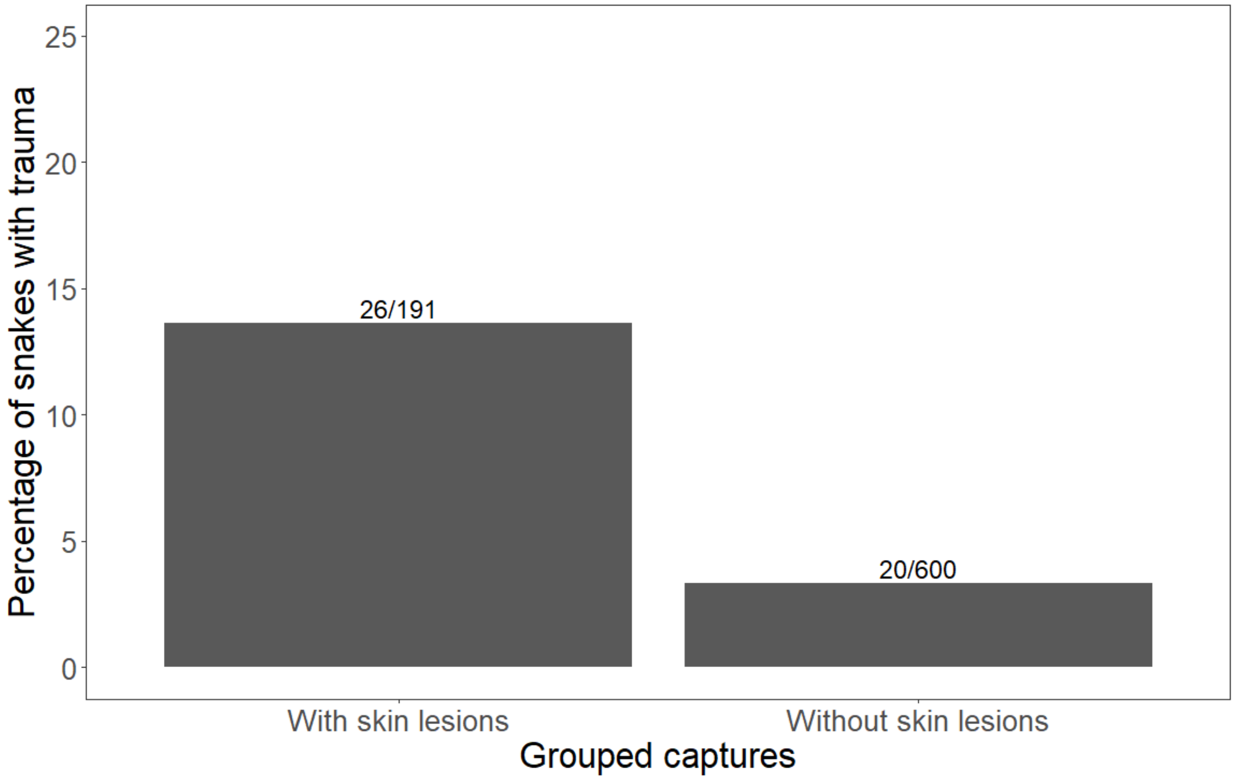


Figure 3.17: The percentage of barred grass snakes (*Natrix helvetica*) observed with skin injuries consistent with trauma, captured 2019-2021 inclusive, grouped with and without detected skin lesions. The y-axis has been set to a maximum of 25, so that the bars can be read more easily.

3.4.6 Presence of a food bolus

There was no significant difference between the presence of a food bolus in captured *N. helvetica* associated with the presence or absence of skin lesions, $\chi^2 = 0.13$, df = 3, p = 0.72.

Table 3.5: The frequency of individual barred grass snakes (*Natrix helvetica*) captured with a detectable food bolus, grouped by year of capture, and whether the snake had detectable skin lesions.

Year	**With skin lesions**		**Without skin lesions**	
2019	Present	28 (41.2%)	Present	112 (49.3%)
	Absent	40 (58.8%)	Absent	115 (50.7%)
2020	Present	10 (33.3%)	Present	79 (38.3%)
	Absent	20 (66.7%)	Absent	127 (61.7%)
2021	Present	25 (36.8%)	Present	76 (52.1%)
	Absent	43 (63.2%)	Absent	70 (47.9%)

3.4.7 Scaled mass index of snakes with skin lesions

The SMI was calculated across the three-year period 2019-2021 and compared between snakes with and without detected skin lesions (Fig. 3.18). There was a significant increase in the SMI of snakes with skin lesions in 2019 (One-way ANOVA, F(1, 312) = 21.27, p =

<0.01), however no significant difference was observed between the groups in 2020 (One-way ANOVA, F(1, 241) = 0.03, p = 0.86), or in 2021 (One-way ANOVA, F(1, 232) = 1.51, p = 0.22). When using the combined data across all three years, there was a significant difference in SMI with snakes with skin lesions having greater values than those without (One-way ANOVA, F(1, 788) = 16.33, p = <0.01).

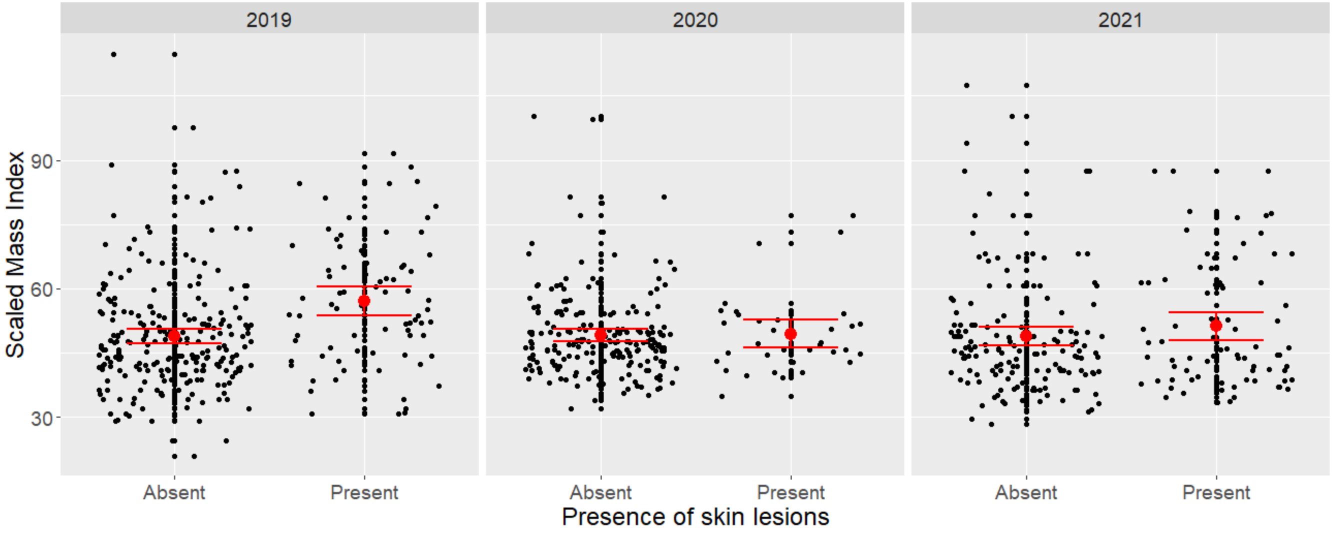


Figure 3.18: A comparison of the scaled mass index of barred grass snakes (*Natrix helvetica*) with and without skin lesions for 2019, 2020 and 2021. The large red dots indicate the mean, with bars representing 95% confidence intervals of the mean. Note that there is a large degree of overlap of points around the means, with points stacked on top of one another.

3.4.8 Comparisons between recaptures

The average severity score (including recaptures) in 2019 was 8.8, for 2020 was 8.2, and 8.1 for 2021. Through the use of recapture data, there is the opportunity to examine the progression of skin lesions in individual *N. helvetica* through time. A total of 54 recaptures throughout the three years of 36 individuals was recorded, with an increase in score severity between paired capture intervals in 33 of these cases, and a decrease in 21 cases (Fig. 3.19). The severity of skin lesions in some individuals remained relatively constant (i.e. within 1-3 points) over the course of a couple of months, whereas in others a greater difference in score (i.e. >4 point difference), was observed over a period that varied from 13 to 84 days. All of the paired observations presented below, were seen within the same year.

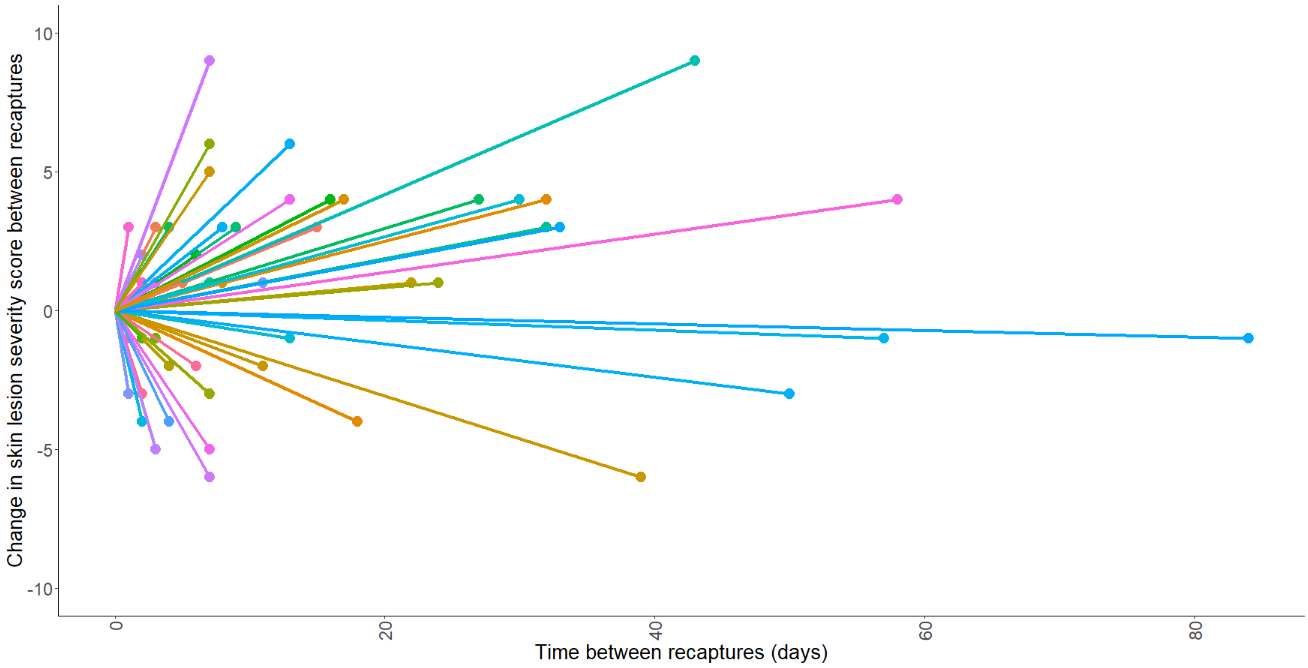


Figure 3.19: A graphical representation of the change in severity score between the initial capture of 36 barred grass snakes (*Natrix helvetica*), and a subsequent recapture within the same year (n = 54). Each colour represents a different individual snake, and the slope of the line indicates the change in severity score through time. 18 snakes were caught on ≥ three occasions: change in skin lesion score between within-year paired captures are represented in two (or more) lines of the same colour.

3.4.9 Modelling predictors of skin lesions

Binomial Generalised Linear Mixed Models were used to test for statistical association between the presence of skin lesions, and the variables measured in the grass snakes, described above. The presence (or absence) of skin lesions was considered the dependent variable, with the independent categorical variables comprising sex, capture month, stage of slough cycle, and the presence of skin injury consistent with trauma. The only continuous variable used was the snout-to-vent length of captured grass snakes. These models were constructed using the data taken from the first capture of each snake caught each year from 2019-2021. Due to the fact that some snakes were recaptured across multiple years, individual snake was used as a random effect in the models. The Akaike Information Criterion (AIC) was used to assess and rank the fit of each model (Akaike, 1974).

The results of these models are displayed below in Table 3.6, along with the delta AIC, weight, log likelihood and degrees of freedom: month, sex, slough cycle, snout-to-vent length and trauma are in the models with the highest scores, which further helps to support the results of the univariate analyses above (excluding sex).

Table 3.6: A summary of Generalised Linear Mixed Models used to determine which factors are associated with the occurrence of skin lesions in barred grass snakes (*Natrix helvetica*), ranked by their AIC score. Also presented at the degrees of freedom, delta AIC, weight, and log likelihood.

Predictor	**Degrees of freedom**	**AIC**	**ΔAIC**	**Weight**	**Log likelihood**
Month + Sex + SVL + Slough Cycle + Trauma	12	602.92	0.00	0.79	-289.25
Month + Sex + SVL + Slough Cycle	11	605.51	2.59	0.21	-291.58
SVL + Slough Cycle + Trauma	7	620.40	17.59	0.00	-303.13
Slough Cycle + SVL	6	621.11	18.19	0.00	-304.50
Month + SVL	7	643.80	40.89	0.00	-314.83
Sex + SVL	4	656.76	53.85	0.00	-324.36
SVL + Trauma	4	657.59	54.67	0.00	-324.77
SVL	3	658.86	55.94	0.00	-326.41
Month + Slough Cycle	9	727.66	124.74	0.00	-354.71
Month + Trauma	7	736.83	133.91	0.00	-361.34
Slough Cycle + Trauma	6	745.89	142.97	0.00	-366.89
Month	6	754.02	151.10	0.00	-370.95
Sex + Month	7	754.15	151.23	0.00	-370.00
Slough Cycle	5	758.18	155.26	0.00	-374.05
Sex + Slough Cycle	6	760.03	157.11	0.00	-373.96
Trauma	3	780.09	177.18	0.00	-387.03
Sex + Trauma	4	780.74	177.82	0.00	-386.34
Food	3	791.53	188.61	0.00	-392.75
Null model	2	793.13	190.22	0.00	-394.56
Sex	3	794.23	191.31	0.00	-394.10

Table 3.7: The generalised variation inflation factor (GVIF) for each of the covariates within the most supported model described above in Table 3.6.

Factor	GVIF	Degrees of freedom
Month	1.48	4
Sex	1.07	1
SVL	1.19	1
Slough Cycle	1.48	3
Trauma	1.07	1

To ensure that there were no confounding variables within the most supported model, the generalised variation inflation factor (GVIF) was investigated (Table 3.7). This indicated that there were no significant correlations between the covariates as the scores were between 1 and 2. When comparing the random effect of individuals in the most supported model, to a fixed factor generalised linear model (the null model), there was a significant difference between the two ($\chi^2 = 211.88$, df = 11, p = <0.01) which indicates that the inclusion of the random effect based on individual is an important factor for the above GLMMs. Inspection of the significant parameter estimates in Table 3.8 indicates the following: the prevalence of skin lesions was lower in July than in the baseline month of May, but the other months did not differ from May. Males had a slightly higher prevalence of lesions than females, and the presence of lesions increased with snake size. Fewer snakes displayed lesions in Phase 0 of the slough cycle than in the other phases. Skin lesions also occurred in slightly more snakes with trauma than those without.

Table 3.8: Parameter estimates, standard errors, z-values, and p-values for the most supported generalised linear mixed model described above (Month + Sex + SVL + Slough Cycle + Trauma). ‘May’ is used as the baseline for comparing variation between months. Significant results are marked with an asterisk.

Parameter	Estimate	Standard error	z-value	p-value
(Intercept)	-3.83	0.64	-6.03	<0.01
June	0.53	0.35	1.52	0.13
July	-0.79	0.32	-2.49	0.01*
August	-0.62	0.39	-1.59	0.11
September	-0.88	0.49	-1.76	0.07
Sex: Male	0.46	0.22	2.09	0.04*
SVL	0.07	0.01	9.22	<0.01*
Slough cycle: Phase 0	-1.40	0.52	-2.72	<0.01*
Slough cycle: Phase 1	-0.11	0.54	-0.21	0.83
Slough cycle: Phase 2	-0.01	0.56	-0.02	0.98
Trauma: Present	0.81	0.37	2.18	0.03*

3.5 Discussion

Skin lesions with an appearance consistent with ophidiomycosis were found to be prevalent throughout the sampling period in the study population of barred grass snakes. Overall, the prevalence of skin lesions across the three year period of survey was 25.5% (191/750) of individual snakes examined. The most common skin lesions characteristics seen in the captured *N. helvetica* were discolouration, crusting, and scale margin erosion. These had previously been described by Franklinos *et al*. (2017) for severe cases of ophidiomycosis seen in *N. helvetica* examined *post mortem*. These results represent the first field data for this species, which contrast markedly with published observations of wild snakes in North America where facial deformities and swelling are not uncommon (Allender *et al*., 2015; Lorch *et al*., 2015).

Studies completed in other snake species in North America have shown that there is high variability in the prevalence of snakes within a population with skin lesions testing rtPCR-positive for *Oo*, based on factors such as the habitat utilised by the snakes (and whether this is favourable to *Oo*), and seasonality (McKenzie *et al*., 2018; Dillon *et al*., 2022). This varies from 83.5-100.0% in aquatic species such as *Nerodia sipedon* (17/17) and *Regina septemvittata* (61/73; McKenzie *et al*., 2018), to 0.0-100.0% in terrestrial species *Opheodrys aestivus* (0/1), *Thamnophis sirtalis* (4/5), and *Lampropeltis getula* (7/7) as examples (Allender *et al*., 2016; McBride *et al*., 2015; McKenzie *et al*., 2018). However, interpretation of any interspecific variation is limited by the small sample size for many of these species.

The findings of this study with a high percentage of skin lesions testing rtPCR-positive for *Oo* are comparable with those from snake species (such as *Nerodia sipedon* and *R. septemvittata*) with similar ecological niches in aquatic habitats from North America (McKenzie *et al*., 2018). Dillon *et al*. (2022) noted that exposure to *Oo* is likely linked to habitat type, with marsh habitats having the highest probability of infection in the eastern fox snake (*Pantherophis vulpinus*), in Ontario, Canada. As *N. helvetica* (and other closely related species in Europe) are semi-aquatic predators, these findings may explain why ophidiomycosis does not yet appear to affect snakes from drier habitats that may co-exist with *N. helvetica* at the landscape scale, such as the European adder (*Vipera berus*).

The distribution of skin lesions observed in affected *N. helvetica* was relatively even across the body length, and less common on the head and tail (i.e. quarter 4), but with notable variation on the body surface occurring predominantly on the ventrum. This is likely explained since the ventrum has greatest contact with the ground, whereby it is more plausibly exposed to the fungal spores of *Oo* within the environment. A similar ventral distribution of skin lesions has previously been reported by Chandler *et al*. (2019) in the eastern indigo snake (*Drymarchon couperi*) in Georgia, USA. Scarification of the *stratum corneum* has been noted to facilitate infection in challenge studies (Lorch *et al*., 2015), with mechanical abrasion caused by a snake navigating its environment, likely to have a similar result.

This study was successful in the detection of *Oo* DNA in the majority of cases where *N. helvetica* had skin lesions consistent with ophidiomycosis, from swabs, skin sloughs, and following PME of carcasses from the study site. These samples also allowed for the detection and identification of *Oo* through histology and culture (Ladner *et al*., 2022). Whilst skin lesion appearance for ophidiomycosis is not pathognomonic, the characteristics and distribution of skin lesions in live animals examined as part of this study were similar to those in *N. helvetica*, with confirmed ophidiomycosis at PME (Franklinos *et al*., 2017).

The high percentage of rtPCR-positive skin lesions supports the hypothesis that ophidiomycosis is their primary cause, as well as a very low percentage of rtPCR-positive results from snakes without skin lesions. False negative rtPCR results from snakes with ophidiomycosis could occur for a variety of reasons, for example when superficial skin swabbing did not permit adequate sampling of cases where the fungi has invaded deeper tissues. Whilst it is likely that there may be multiple aetiologies of skin lesions for snakes that tested rtPCR-negative for *Oo*, based on these study results, using skin lesion occurrence as a proxy for ophidiomycosis appears appropriate. There was also a significant association between the Ct value and the severity score of skin lesions, which indicates that more *Oo* is present in cases with more severe skin lesions and also supports the hypothesis that ophidiomycosis is their aetiology.

For the minority of cases (20.4%) where *N. helvetica* with skin lesions tested rtPCR-negative for *Oo* DNA, it is also plausible that other potential infectious agents were the cause, such as *Chrysosporium* spp. (Bertelsen *et al*., 2005; Bowman *et al*., 2007; Cahañes *et al*., 2014) and *Paranannizziopsis* spp. (Rainwater *et al*., 2019), which can present similarly clinically in reptiles such as wild snakes. Therefore, disease surveillance should continue to investigate the aetiology of skin lesions, using a combination of ancillary diagnostic tests, to gain a better insight into range of aetiologies of skin disease in wild European snake species. This would most likely be achieved through post-mortem examination of deceased snakes, with input from a multi-disciplinary team of wildlife health professionals.

For those snakes where rtPCR-positive skin lesions were present, these can be categorised as apparent ophidiomycosis, following the terminology developed by Baker *et al*., (2019). It was not feasible or ethical to conduct histopathological analysis on samples (that would require collection of biopsy and/or scale clips) from all of the affected snakes, so we can only conclude that those 6 cases (from a carcass and 5 skin sloughs) where both rtPCR and histopathological examination was possible, the carcass and three sloughs had confirmed ophidiomycosis, one slough had possible/apparent ophidiomycosis, and one slough had *Oo* present. The small number of rtPCR-positive swabs from *N. helvetica* without detected skin lesions may be explained by sub-clinical infection, or environmental contamination, and are classified as *Ophidiomyces* present (Baker *et al*., 2019).

It is noteworthy that none of the snakes with detected skin lesions, even those classified as severe, exhibited altered patterns of behaviour during capture and handling, consistent with systemic ill health, or a moribund state. These findings contrast with those previously reported in affected snakes in North America (Lorch *et al*., 2016). This may be a consequence of survivorship bias, with the more severely affected snakes dying away from ACOs therefore going undetected. However, given the severity scores of affected snakes were generally quite low, it is likely that this is the true relationship between *N. helvetica* and ophidiomycosis. Therefore, direct mortality caused by ophidiomycosis in *N. helvetica* is likely to be low, although further research is required to confirm this. Radio-tracking of affected snakes could provide a reliable method of tracking individuals, and therefore monitoring their disease progression and survival outcome.

Regarding the lesion severity scores, there was a skew towards mild cases. These mild skin lesions may be consistent with hibernation sores or blisters, previously recorded in snakes from North America (Lorch *et al*., 2016), which form during the snake hibernation period. If fungal infection is superficial, *Oo* may be shed when the snake sloughs in the spring. Harding *et al*. (2022) when investigating the presence of *Oo* in North American water snakes (*Nerodia* spp.) also found that adults had the highest prevalence of PCR-positive skin lesions. The findings presented in this chapter with increased body condition index of snakes with skin lesions contrast with published studies where adverse effects or no change of body condition was observed with ophidiomycosis (see Allender *et al*., 2016; McCoy *et al*., 2017); however, this may be a sampling artefact, given the majority of snakes with skin lesions were adults. In addition, there was no evidence of anorexia (inferred through absence of food bolus) associated with skin lesions. Collectively, these findings suggest that if there are adverse impacts on the host health, then they are likely to be indirect, or due to a bias in our large sample of adults relative to other size classes.

Based on challenge studies, it is likely that snakes with skin lesions may bask out in the open more often following infection (Tetzlaff *et al*., 2017), and therefore increase their risk of predation. This has also been observed in wild populations of *S. miliarius*, with snakes with apparent ophidiomycosis displaying higher frequencies of thermoregulatory postures than those clinically healthy snakes (Lind *et al*., 2022). The higher occurrence of trauma in those snakes with skin lesions suggests that these snakes may have come into contact with potential predators, and escaped, more frequently than those snakes without skin lesions. Snakes suffering from trauma (and the subsequent stress) may also lead to them being immunocompromised, and more likely to succumb to ophidiomycosis. While puncture wounds are highly suggestive of predation, the cause of tail damage and lacerations is less certain, although may still be linked to predation (Gregory & Isaac, 2005; Santos *et al*., 2011). Affected snakes may also have spent longer periods of time basking in more risky scenarios in order to maintain the higher metabolism needed to promote an increased sloughing frequency (Lorch *et al*., 2015), with a higher proportion of snakes with skin lesions of those caught out in the open, compared to those underneath ACOs.

Ophidiomycosis may promote an increased rate of sloughing which may help infected snakes to clear infection (Lorch *et al*., 2015; Lind *et al*., 2022), but this can also inadvertently lead to higher rates or predation due to snakes being unable to see for comparatively longer periods of time, or having to slough in more risky microhabitats (King & Turmo, 1997). We found that skin lesions were more likely to be associated with the later stages of the slough cycle compared to those without, which is likely a consequence of affected snakes sloughing more frequently.

There was a slight but significant difference between the occurrences of skin lesions between the two sexes when analysing the data using the multivariate models, with males having a higher prevalence of skin lesions. These results contrast with Lind *et al*., (2022), who found that there was no link to sex or reproductive status between adult pygmy rattlesnakes (*Sistrurus miliarus*), and the likelihood of being PCR-positive for *Oo* DNA. Males are more active while they search for suitable females, whereas females tend to search for egg-laying sites (Madsen, 1984). The results were different when using the univariate analyses, which may reflect the marginal significant result in the multivariate analysis. However, the multivariate models control for the influence of other predictors in the same model, when compared to the univariate analysis, providing a more robust result.

Adults were most often found to have skin lesions which may be a consequence of them sloughing less frequently than younger snakes, and the larger surface area to contract fungal infection from the environment. In challenge studies (Lorch *et al*., 2015), skin lesions of ophidiomycosis started to appear within as little as four to eight days post-infection with *Oo*. For sub-adult *N. helvetica* which may only slough four times in a year (Brown, 1956), and less often in adults, clearing infection through increased frequency of shedding may not be feasible, especially if the fungal hyphae have penetrated beneath the dermis.

High frequencies of skin lesions in *N. helvetica* coincided with their peak active season throughout May and June, with approximately 30% of snake captures with skin lesions. Our data indicate a strong seasonality of ophidiomycosis, linked to the activity of *N. helvetica*. This is a similar outcome to other studies where skin lesions are more prevalent in snakes following emergence from hibernation (Lorch *et al*., 2016; Dillon *et al*., 2022). Depending on the sampling year, skin lesions peaked in May as snakes were encountered post-hibernation, with infected snakes becoming less frequently encountered after that time period. This may be linked to an increase in temperature, which may aid in enhancing immune function and clearing infection, or it may be more closely correlated to the hibernation and slough cycle of snakes.

Through the use of serial recaptures, this study found evidence of progression, apparent healing and lesion recurrence among individual snakes. For cases with apparent recurrence, fungal hyphae may have remained in the skin (either by penetrating deeper layers or through the presence of improperly sloughed skin), leading to subsequent development of further skin lesions (Lorch *et al*., 2015). Snakes can undergo changes in seasonal variability of infection with ophidiomycosis as indicated by change in skin lesion appearance (n = 2; Duffus *et al*., 2022), which can be monitored through the use of recaptures. This study expands on the work previously completed by Duffus *et al*. (2022), by following a greater number of individuals (n = 36), over a more substantial period of time. Some of the affected snakes had a relatively consistent appearance of skin lesions over long periods of time between captures (up to 84 days), whilst others had increased or reduced skin lesion severity scores over an interval of 1-20 days.

Through the use of generalised linear mixed models, we identified that a number of factors influence the likelihood of skin lesion occurrence, such as the month of capture, sex of snakes, slough cycle, the snout-to-vent length of the affected individual, and the presence of trauma. A number of these factors were also supported through the use of univariate analyses, most notable of which were the stage of the slough cycle and presence of trauma. If further monitoring of this population of *N. helvetica* (or others) were to be undertaken to determine whether snakes were infected with ophidiomycosis, these could be important considerations in helping to target those individuals that are most likely to have skin lesions consistent with ophidiomycosis, such as early emerging adults, if restrictions such as time and money meant that a study such as the one described within was not feasible.

Research has shown a strong correlation between rtPCR-positive sloughs, and apparent ophidiomycosis in *N. sipedon* (Dibaji *et al*., 2021). However, these sloughs were collected from captively maintained wild-caught individuals, with a known history of ophidiomycosis. We also found that sloughs collected in the field could be used for *Oo* detection using rtPCR. The lower percentage of *Oo* detection in skin sloughs with lesions in this study, as compared with Dibaji *et al*. (2021), could be explained by a range of factors. Sloughs we collected in the field may have been in comparatively poor condition, with likely breakdown of DNA due to the presence of moisture and keratinophilic saprophytes, potentially leading to false negative test results. All skin slough samples were also tested using the internal control PCR, with zero negatives. Alternatively, since the slough lesions were less characteristic than the skin lesions in live snakes and comparatively non-specific in appearance, it is plausible that there were multiple causes other than ophidiomycosis.

Whether ophidiomycosis presents in the same way in other *N. helvetica* populations (or those of other *Natrix* species) throughout Europe is yet to be seen. The findings of this study will help to inform other related research projects across both sites and species in Europe, by providing a framework to determine if the characteristics associated with ophidiomycosis outlined within, are representative across wild snake populations throughout the continent. To date, small numbers of snakes in Germany and Poland have tested rtPCR positive for *Oo* DNA, but wider surveillance is needed (Blanvillain *et al*., 2022).

The combined results from histopathology and rtPCR outlined within indicate that skin lesions can reasonably be used as a proxy of ophidiomycosis in *N. helvetica* at the study site. In Chapter 4, we estimate the apparent annual survival and detectability of *N. helvetica* with and without skin lesions, and use this to infer the impact of ophidiomycosis on host health.

3.6 Acknowledgements

We are grateful to the landowners (Angela and Louisa Knapp) of Watermill Broad Nature Reserve for granting us access to complete this study, to the previous Master's students (Angela Winnett [2016], Helena Marsh-Williams [2017], and Sargai Sha [2018]) from the University of East Anglia (UEA)) for sharing data from their fieldwork on barred grass snakes (*Natrix helvetica*) at the study site, and Professor Iain Barr from the UEA for his role as a supervisor to these students. This research would not have been possible without help from Dr Jeffrey M. Lorch from the National Wildlife Health Center, U.S. Geological Survey, Madison, Wisconsin. We thank Dr Julia Rodriguez-Ramos Fernandez DVM DiplECZM (WPH) DiplACVP MRCVS from IDEXX laboratories, who conducted the histopathological examinations, and Kevin Hopkins, Matthew Perkins, and Katharina Seilern-Moy from the Institute of Zoology, ZSL, for their supervision, guidance and troubleshooting assistance with the molecular diagnostics.

3.7 Supplementary Materials

3.7.1 Field work biosecurity protocol

When the ACO is lifted, the snakes underneath will be caught and placed in individual large freezer bags. These will be tied loosely so that snakes cannot escape but there is enough air/air circulation to ensure that they do not suffocate. Whilst in the bags, the snake will be checked for visible signs of skin lesions. If lesions are present the snakes will be swabbed as they are taken out of their bags before processing continues. After being weighed, the snout-to-vent length (SVL) of each snake will be measured using a fibreglass measuring tape. Each snake will then be laid upside down on a sheet of white acrylic and their anterior ventral scale pattern will be photographed for identification purposes.

During the handling process, care will be taken to avoid contamination of equipment (e.g. camera) and external swab packaging with dirty gloves. Nitrile gloves will be changed between the handling of each snake and all plastic bags used to contain snakes will be disposed of after use.

Both the acrylic sheets and the tape measures (as well as any other equipment that has come into contact with the snakes) will be disinfected between use, using antifungal spray and wipes (Dettol All-in-One Disinfectant spray; dimethyl benzyl ammonium saccharinate 0.1 % active ingredient), 10 minutes contact time following Rzadkowska *et al*., 2016). The equipment will be rinsed with tap water and air-dried to ensure the surfaces are safe to use upon handling of the next snake. Only one side of the acrylic sheets will be used at a time to allow the other side to be disinfected in the event of capturing multiple snakes under the same refugia.

Once the survey has been completed, all re-usable pieces of equipment will be washed down and sterilised with a 1% Virkon S solution (using a spray bottle, 10 minute minimum contact time) and rinsed with tap water before being left to air-dry in direct sunlight. The equipment will be scrubbed with a sturdy brush and soapy water to remove any dirt/mud (if necessary). Any excess Virkon will be left for 5 days to allow it to become inert before disposal in the local environment or down a drain. All Virkon treatment will be carried out at a designated area of the site to reduce the risk of damage to the surrounding environment (note Virkon is toxic in aquatic environments so must not enter water bodies). Similarly, cloth bags will be disinfected with 1% Virkon S for at least 10 minutes, rinsed with clean water and left to air dry: as an extra precaution following heavy soiling, the cloths bags will also be washed at 60˚C on a regular basis.

All waste produced by the activities of surveying snakes for the duration of the project will be collected and stored in a secure bin within the Attenborough Hut, ready to be collected and disposed of appropriately as clinical waste at ZSL London Zoo. All equipment will be stored in a secure container in the hut between surveys on site. Dedicated footwear and overalls will be used at the site, which shall not be removed until the end of the survey period when they will be disinfected before removal off site.

3.7.2 Skin lesion definition and classification system in barred grass snakes (*Natrix helvetica*)

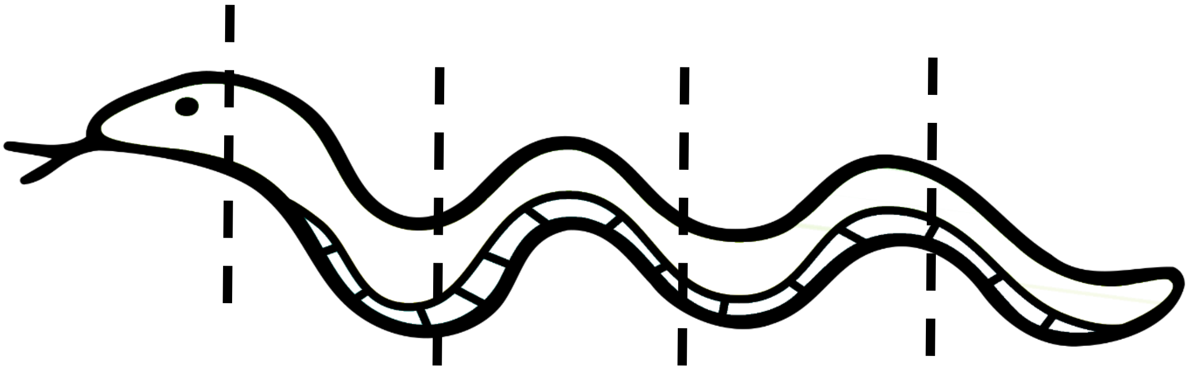
The following protocol is designed to capture as much information about the skin lesions present on a snake in order to fully describe them and to characterise their severity.

Classification:

Are skin lesions visible?

Yes = 1, No = 0

If present, what is their anatomical location?



- Head
- 1st ¼ (dorsal, ventral or lateral)
- 2nd ¼ (dorsal, ventral or lateral)
- 3rd ¼ (dorsal, ventral or lateral)
- 4th ¼ (dorsal, ventral or lateral)

When in the field, the entire snake will be checked for the presence of skin lesions. Locations are not mutually exclusive as lesions may be present in more than one location. It is important to ensure that the location of skin lesions is established using the above diagram.

How many skin lesions are there?

A count of the total number of skin lesions present. If skin lesions are too numerous to count, indicate where the total is an estimate (or annotate with TMTC).

What is/are the colour(s) of the skin lesion(s)?

- Black
- Dark brown
- Red
- Tan
- Yellow
- Other (specify)

Colours are not mutually exclusive; more than one colour can be used to describe the lesions present on a snake.

What is/are the character(s) of the skin lesion(s)?

- Crusting/scab: where scale(s) has/have become thickened and/or hardened. Yes = 1, No = 0
- Discolouration: where scale(s) is/are a different colour to adjacent scales of the same type. Yes = 1, No = 0
- Distorted scales: scale(s) that appears misaligned with surrounding scales and/or are misshapen. Evidence of scarring may be apparent. Yes = 1, No = 0
- Dysecdysis: Abnormal sloughing with evidence of skin retention. Yes = 1, No = 0
- Scale margin erosion: Irregular prolife of scale margin(s) due to tissue loss/erosion. Yes = 1, No = 0
- Swelling: Swelling of scale(s), raised in profile with visible doming. Yes = 1, No = 0
- Ulceration: The complete loss of scale(s) or dermis that expose the underlying tissues. Yes = 1, No = 0

Each lesion is to be described in terms of the characteristics above, or summarised where multiple lesions of a similar appearance are present. It is important to note when skin lesions of variable appearance are present on the same individual. The descriptions are not mutually exclusive.

What is/are the size of the skin lesion(s)?

The size of each lesion is to be estimated in the field or measured with digital Vernier calipers (if time permits) in millimetres. Lesions are also to be photographed alongside a scale bar so that precise measurements can be made later, with software such as ImageJ. There is a priority to collect data on lesions with multiple characters as well as the largest lesion present in order to help inform the severity of infection.

Note: Due to time constraints, not all of the lesions may be measured within the given handling time. It is important to measure as many of the lesions as possible within the allocated time as smaller lesions may have different characters to larger ones.

Trauma

Traumatic injuries may be detected in grass snakes (e.g. laceration, puncture wound). To differentiate skin abnormalities where the aetiology is strongly suspected to be the result of trauma, from lesions likely due to infectious disease (i.e. ophidiomycosis), the following information is recorded.

Are signs of trauma visible?

Yes = 1, No = 0

If present, where on the body is the trauma?

- Head
- 1st ¼ (dorsal, ventral or lateral)
- 2nd ¼ (dorsal, ventral or lateral)
- 3rd ¼ (dorsal, ventral or lateral)
- 4th ¼ (dorsal, ventral or lateral)

What is/are the character(s) of the trauma?

- Puncture wound
- Tail tip missing
- Laceration
- Abrasion
- Other

3.7.3 Skin lesion severity scoring system

Based on the clinical signs of skin lesions observed in the barred grass snake the following scoring system was developed to grade skin lesion severity, adapted from Baker *et al*., (2019). Points are assigned based on highest the possible value in each category:

1. Lesion type: discolouration/swelling = 1 point; crusting/distorted scales/scale margin erosion (<5 mm) = 2 points; scale margin erosion (>5 mm) = 3 points; dysecdysis/ulceration = 4 points

2. Lesion location: dorsal, lateral or ventral surface of body (one of the options) = 1 point; dorsal, lateral or ventral surface of body (multiple options) = 2 points; head (but not around mouth or eyes) = 3 points; head (around mouth or eyes) or cloaca = 4 points

4. Lesion number: single lesion = 1 point; 2–4 lesions = 2 points; 5–9 lesions = 3 points; 10 or more lesions = 4 points

4. Lesion coverage (total size (diameter of round lesions, length of linear lesions) of all lesions present): <10 mm = 1 point; 10–50 mm = 2 points; 50-100 mm = 3 points; >100 mm = 4 points

Based on our observations in barred grass snakes we categorise 4 points as very mild, 5–8 points as mild, 9-12 points as moderate and 13-16 points as severe skin lesions.

Chapter 4. Comparison of survival and detectability in the barred grass snake (*Natrix helvetica*) in relation to skin lesions

4.1 Abstract

A population of the barred grass snake (*Natrix helvetica*) was studied over the period 2015 to 2021 in order to i) calculate survival rates and ii) relate variation in survival probability to a range of potential drivers, in particular the effects of the emerging infectious disease ophidiomycosis. Over the seven years of data collection, 1935 captures of 1143 individual snakes were made by five different surveyors. Through the use of Cormack-Jolly-Seber Models, snakes with skin lesions used as a proxy of ophidiomycosis demonstrated lower apparent annual survival (φ = 0.224-0.225) and higher detectability (p = 0.693-0.721) than snakes without skin lesions (φ = 0.531-0.639; p = 0.08-0.163). Both apparent annual survival and detectability probabilities varied over time. Neither sex nor snout-to-vent length of individuals was related to survival or detectability. Jolly-Seber models were used to produce population estimates, which fluctuated between 323 to 780 (SE: 66.15-179.67) individuals in the population across the seven years of surveys. These results demonstrate the ability to obtain a survival rate in a long-lived species that is challenging to detect in the field and suggest that *N. helvetica* populations in the UK may be impacted by ophidiomycosis.

4.2 Introduction

Approximately, one fifth of the world's reptile species are currently listed as Data Deficient by the IUCN (Böhm *et al*., 2013; Cox *et al*., 2022). The monitoring of wildlife populations is essential to inform effective conservation measures and the success of interventions (Yoccoz *et al*., 2001; Nichols & Williams, 2006). Identifying the causes of declines observed is a necessary precursor to threat mitigation and, ultimately, prevention of extinction (Lande, 1998).

The main processes determining the size of a population are reproduction, survival and immigration/emigration. Survival is a key driver of population size for long-lived organisms, such as birds (Sæther & Bakke, 2000) and some reptiles (Webb *et al*., 2002), potentially including the barred grass snake (*Natrix helvetica*), which can live for 15 years or more (Beebee & Griffiths, 2000). Changes in abundance may take a long time to be expressed, whereas changes in survival rates may be exhibited more quickly (Gibbons *et al*., 2000), rendering the latter a more sensitive and therefore effective monitoring tool. Survival monitoring also helps to overcome the fact that that abundance of reptiles is generally hard to assess directly due to their low detectability, the product of low population densities and cryptic behaviour (Durso *et al*., 2011). Density-dependent factors regulating wild snake populations include food availability, density of predators, and disease risk. Whereas density-independent factors are those such as environmental quality or the risk of flooding, which are not influenced by population size (Edward & Edwards, 2011).

Disease is a factor that may influence survival in wildlife populations by leading to morbidity and/or mortality of affected animals, through direct impact on individual health or indirect mechanisms such as increased rates of predation (Lorch *et al*., 2016). Ophidiomycosis (caused by the pathogenic fungus *Ophidiomyces ophidiicola*; hereafter *Oo*) causes skin lesions to develop but their significance to host health may vary according to location on the body: for example skin lesions around the eyes and mouth of affected snakes, may impact their ability to feed or hunt (Allender *et al*., 2015; McBride *et al*., 2015; Lorch *et al*., 2016). Skin lesions may also occur around the cloaca, impacting on the snake’s ability to breed or excrete waste efficiently (Grisnik *et al*., 2018). A systemic infection, with multiorgan involvement, may result in sufficient tissue damage to kill the host (Dolinksi *et al*., 2014). The current evidence suggests, however, that wild snakes tend to die from secondary complications of ophidiomycosis, such as immunosuppression and secondary bacterial infection or indirect mortality due to predation, rather than from direct fungal damage itself (Lorch *et al*., 2016).

Another potentially important factor influencing the survival of *N. helvetica*, is that of predation. Predators of *N. helvetica* include the carrion crow (*Corvus corone*) and magpie (*Pica pica*) among other birds (Madsen, 1987), and mammals such as European badgers (*Meles meles*), red foxes (*Vulpes vulpes*), and domestic cats (*Felis catus*), although grass snakes do not form a substantial part of the diet of these mammalian species (Beebee & Griffiths, 2000). As predators themselves, populations of snakes are also reliant on prey availability; if there are sudden and prolonged crashes in prey populations, then this is likely to have similar consequences in snakes (Zipkin *et al*., 2020). In some populations, roads may act as a major contributing factor to mortality in *N. natrix*, which is also likely in *N. helvetica* (Borczyk, 2004; Ciesiołkiewicz *et al*., 2006).

Capture-mark-recapture (CMR) is an approach frequently utilised to estimate survival and detectability (Horten & Letcher, 2008) via the recapture of marked individuals over both short-term and long-term intervals (Katz *et al*., 2013; Zylstra *et al*., 2013). Cormack-Jolly-Seber (CJS) models can be used to estimate these parameters on the basis of recapture data, where individuals naturally possess unique markings (Gatto *et al*., 2018; Moro & MacAulay, 2014; Sacchi *et al*., 2010) or can be fitted with a device (such as flexible elastomer implants) allowing distinct identification (Major *et al*., 2020; Du Feu *et al*., 2016). Barred grass snakes are well suited to CMR studies due to the patterns of markings on their ventral scales, which are unique to each individual (Carlström & Edelstam, 1946).

N. helvetica populations in Great Britain and the Channel Islands have been monitored previously via the use of artificial cover objects (ACOs), but little is known about their demographic rates (Sewell *et al*., 2015; Ward *et al*., 2017). Using a CJS approach, Sewell *et al*. (2015) estimated the annual survival of a *N. helvetica* population in the East of England to be 0.66 (95% CI 0.543–0.755), but this pre-dates the confirmation of ophidiomycosis in Great Britain (Franklinos *et al*., 2017).

The aim of this study is to investigate a range of factors that influence the annual survival of *N. helvetica*, utilising data from a population in eastern England (see Chapter 2) monitored from 2015 to 2021, with a focus on skin lesions consistent with ophidiomycosis, as a proxy for infection (see Chapter 3). Other factors that potentially influence the survival of *N. helvetica* were also investigated, including mass, sex, and length, of individuals, and the year of capture.

4.3 Materials and methods

4.3.1 Structured surveys

A total of 69 artificial cover objects (ACOs) were evenly distributed across the field site (67 ACOs were used from 2015 to 2018), The ACOs were placed within 2-5 m of their original location each season after being collected for winter storage, depending on vegetation growth and topography, with half of the ACOs being moved and the other half placed in the same location as the previous season. Capture visits were conducted during the summer across the seven years; the period of overlap across all years was May to July, which is when *N. helvetica* tend to be most active following hibernation, and the analysis was restricted to this time period. Capture visits were either undertaken in the morning (between 0900 and 1100 hrs), or in the afternoon (between 1600 and 1900 hrs). Visits were conducted when the air temperature was above 10°C, and when there were no strong winds or heavy rain (>4 mm/hour), which would have reduced the activity of *N. helvetica*. Visits were also avoided when the air temperature was above 30°C, due to the reduced use of ACOs by *N. helvetica* at those temperatures. See Chapter 2 for a more detailed overview of the survey methods used.

4.3.2 Identification of individuals and determination of sex

When encountered, snakes were captured by hand and photographed for identification; whilst in the hand biometric data (body mass, snout-to-vent length, and sex) were also recorded. The unique ventral scale pattern of each snake was photographed to allow for the identification of individuals using the pattern matching software Wild-ID (Bolger *et al.*, 2012). For more information on use of Wild-ID, see Chapter 2. The sex of captured *N. helvetica* was determined by the number sub-caudal scales, or by the presence of a hemipenal bulge (see Chapter 2), allowing the sex of a grass snake to quickly be identified even when they are not sexually mature (Langton, 1989; Gent & Gibson, 1998).

4.3.3 Ophidiomycosis and model assumptions

Over the three-year period 2019-2021, the entire surface of each snake was examined for the presence of skin lesions, although these were usually present on the ventral surface only (see Chapter 3), which were swabbed for later rtPCR analysis for *Oo* detection. Comprehensive examination did not occur during the period 2015-2018, however, and presence of skin lesions was established from images taken to determine snake identity, which were limited to the head and first quarter of the ventral surface. Over the period 2019-2021, 70% of snakes exhibiting skin lesions could have been categorised as diseased through photos of the head and first quarter alone (2019, 72%, N = 92; 2020, 60%, N = 43; 2021 68%, N = 79). To maintain consistency, we therefore only considered snakes with skin lesions present on the head and first quarter when assigning disease status, acknowledging that this is a conservative approach as skin lesions may have been present at the posterior end of affected snakes.

Two modelling scenarios were undertaken, due to the fact that some snakes had varying skin lesion status (i.e. some individuals transitioned between skin lesions being present or absent in between recaptures) between the years they were recaptured (Table 4.3). It is reasonable to assume that infected snakes are able to clear infectious through either behavioural, or immunological means, as has been demonstrated experimentally (Lorch *et al.*, 2015). For the sub-set of snakes that apparently lost skin lesions between captures, however, it is possible that they were not detected due to the individual having recently sloughed. The first modelling scenario (Scenario 1) assumed that once skin lesions had been detected, the disease status in the affected individual did not change thereafter, regardless of any subsequent observations to the contrary. The second (Scenario 2) assumed that recovery was possible and that if snakes initially displaying lesions were recaptured in subsequent years with no lesions detected, they were truly disease-free.

Given the assumptions made about skin lesions, Scenario 1 is likely to retain recovered snakes within the ‘diseased’ category, thereby reducing the detectable relationship between skin lesions and survival in a conservative manner. Scenario 2 potentially corrects for this source of error by assuming that recovery is possible between recaptures, but may still incorrectly classify a subset of diseased snakes as ‘recovered’ if the stage of the slough cycle influences lesion detectability.

4.3.4 Creation of the survival matrices

Capture histories were compiled with each uniquely-numbered individual allocated ‘1’ (captured) or ‘0’ (not captured) on any sampling occasion in a given year (see Supplementary Information 4.7.1) The timing and number of surveys carried out within the May-July focal period varied between years due to different environmental conditions and the observer. To try to account for this disparity, the number of surveys (which were each of roughly equal length, lasting 1.5 hours) completed during this time were added as one of the explanatory variables determining recapture rate. Additionally, models can account for these fluctuations through the time-dependent framework detailed below.

4.3.5 Estimating annual apparent survival

We used time-dependent models and RMark (Laake, 2013), which are flexible in being able to utilise covariates that vary through the duration of a study (Lebreton *et al.*, 1992). The covariates investigated were mass, length, sex, presence of skin lesions, and size class. Within-year survival models could not be used as sampling frequency was not sufficiently consistent through the year, so as an alternative we fitted annual survival models. These annual models contained an additional covariate accounting for the number of surveys conducted within the May to July survey period each year. In all cases, if covariates were found to have little or no positive effect on the outcome of the models (indicated by a high Delta AIC score, with the threshold being >2), they were removed from future iterations, to minimise the degrees of freedom, and reduce the number of parameters within the models.

Interactions between potential suitable covariates were not explored in the final round of modelling, due to the presence of boundary estimates (indicated by estimated survival or detectability values bordering either 1 or 0) in those models including them, outlined above in Table 4.1. Of these covariates, we restricted the analysis to those models specified in Table

4.2 because they were consistently shown to be the most important covariates in the pilot modelling iterations (see Supplementary Information 4.7.2). R version 4.0.3 (R Core Team, 2020) was used throughout the modelling process.

Goodness of fit tests were used to ensure that there was no overdispersion within the data before further progression (see Supplementary Information 4.7.3). The variance inflation factor ($\hat{c}$), was calculated through the R package ‘R2ucare’ (Gimenez *et al*., 2018), to check for transience within the dataset, and for correlation between the variables used. A $\hat{c}$ value of around 1 demonstrates that overdispersion is not present within the dataset, which also indicates a lack of transience. Transient individuals were defined as those that were only caught once, and likely spent a short time at the study site. It was assumed that there may be some transience within the population, given the mobile nature of *N. helvetica* (Madsen, 1984). This was accounted for by using time-dependent models, which have greater flexibility regarding transience. If significant levels of transience were detected within the dataset, then this could be accounted for by estimating the time resident and transient snakes spent within the population, through the use of Test 3.Sm, see Section 4.4.1 for results.

4.3.6 Model selection

The fit of each model was inferred using the Akaike Information Criterion (AIC) to rank models (Akaike, 1974). The ΔAIC metric assigned to each model signifies its explanatory power relative to every other model constructed. A ΔAIC score of between 0 and 2 is indicative of a model that explains substantially more variance than the alternatives, and therefore has a high degree of support (Burnham & Anderson, 2002).

Table 4.1: The Cormack-Jolly-Seber models used to determine which variables influence barred grass snake (*Natrix helvetica*) survival and detectability probabilities, across the initial time-dependent modelling stage. Interactions were trialled at this stage to determine if the data were of sufficient quantity to allow estimates to be made. The model parameter of time indicates the marking duration of snakes, which in this study were at annual intervals.

Parameter	Number of parameters	Model	Interpretation
Annual survival (φ)	1	Dot	Survival of snakes is constant
	1	time	Survival of snakes varies annually
	6	Skin lesions	Survival of snakes varies with the presence of skin lesions
	7	Skin lesions: time	Survival of snakes varies with the interaction between skin lesions and time
	7	Skin lesions: sex	Survival of snakes varies with the interaction between skin lesions and sex
	7	time + skin lesions	Survival of snakes varies annually and the presence of skin lesions
	1	Length	Survival of snakes varies with their length
	7	Length: skin lesions	Survival of snakes varies with an interaction between length and skin lesions
	7	Length + skin lesions	Survival of snakes varies with their length and presence of skin lesions
	7	time + length	Survival of snakes varies annually and with snout-to-vent length
	7	Skin lesions + sex	Survival of snakes varies with the presence of skin lesions and snake sex
Annual detectability (*p*)	1	Survey number	Encounter probability of snakes varies with the number of surveys
	1	Dot	Encounter probability of snakes is constant
	1	time	Encounter probability of snakes varies annually
	6	Skin lesions	Encounter probability varies with the presence of skin lesions
	7	Skin lesions + sex	Encounter probability varies with the presence of skin lesions and snake sex
	7	Skin lesions: sex	Encounter probability varies with an interaction between skin lesions and sex
	7	Skin lesions: time	Encounter probability varies with an interaction between skin lesions and year
	7	time + skin lesions	Encounter probability varies with year and the presence of skin lesions
	1	Length	Encounter probability of snakes varies with their snout-to-vent length
	7	Length + skin lesions	Encounter probability varies with their length and the presence of skin lesions
	7	Length: skin lesions	Encounter probability varies with an interaction between length and skin lesions

Table 4.2: The Cormack-Jolly-Seber models used to determine which variables influence barred grass snake (*Natrix helvetica*) survival and detectability probabilities, within the constrained time-dependent model analysis (Scenarios 1 and 2). The model parameter of time indicates the marking duration of snakes, which in this study were at annual intervals.

Parameter	**Number of parameters**	**Model**	**Biological interpretation**
Annual survival (φ)	1	time	Survival of snakes varies by year
	7	time + skin lesions	Survival of snakes with skin lesions varies by year
	2	Skin lesions	Survival of snakes varies with the presence of skin lesions
Annual detectability (*p*)	7	time + skin lesions	Encounter probability of snakes with skin lesions varies by year

4.3.7 Jolly-Seber models to determine population size

Population estimates of the numbers of *N. helvetica* that used the ACOs across the seven years of study were calculated using Jolly-Seber models (Schwartz, 2001), in the R package 'RMark' (Laake, 2013). These estimates of the population size were made using the same capture history created for the CJS models outlined above.

4.4 Results

Cormack-Jolly-Seber models were successful in producing apparent annual survival and detection estimates for *N. helvetica* across seven years of surveys. The estimates provided below indicate the probability of survival or detectability of an individual within a given year. No snakes changed status between the two states more than once (see Table 4.3).

Table 4.3: The number of recaptured barred grass snakes (*Natrix helvetica*) by survey year, from May to July, with or without skin lesions, and those that did or did not change skin lesion state between recaptures.

	2015	**2016**	**2017**	**2018**	**2019**	**2020**	**2021**
Snakes with skin lesions	60	20	42	41	71	31	54
Snakes which developed skin lesions relative to previous year(s)	NA	4	4	5	14	7	17
Snakes which healed skin lesions relative to previous year(s)	NA	2	1	0	0	2	2
Snakes without skin lesions	78	59	94	103	147	155	110
Total	138	85	141	149	232	195	183

Table 4.4: The AIC for models outlined in Table 4.1 above. Only the scores of the top 10 models have been shown, which helped to influence the constrained models highlighted in Table 4.2. Phi = annual survival; p = annual detectability.

Model	**Parameters**	**AIC**	**ΔAIC**	**Weight**	**Deviance**
Phi(~lesions + time)p(~lesions + time)	14	796.67	0.00	0.44	768.67
Phi(~time)p(~lesions + time)	13	796.88	0.21	0.40	770.88
Phi(~time + svl)p(~lesions + time)	14	798.66	1.99	0.08	770.66
Phi(~lesions + time)p(~time + sex)	14	799.24	2.57	0.06	771.24
Phi(~lesions: time)p(~time + sex)	14	799.54	2.88	0.05	771.54
Phi(~lesions: time)p(~nsurvey)	9	801.06	4.39	0.02	783.06
Phi(~lesions: time)p(~time)	13	801.37	4.70	0.02	775.37
Phi(~lesions + time)p(~time)	13	801.60	4.92	0.02	775.59
Phi(~lesions: time)p(~lesions + time)	14	802.78	6.10	0.01	774.78
Phi(~lesions + time)p(~nsurvey)	9	804.81	8.14	<0.01	786.81

4.4.1 Investigating transience using R2ucare and ĉ

Given the highly mobile nature of *N. helvetica*, some transience was expected, with the evidence of transience within the population. However, the statistical test for transience (ĉ) was not close to significance, $\chi^2 = 5.95$, df =14, p = 0.968.

Test 3.SR, investigating whether there is a capture and 'marking' effect (i.e. whether subsequent survival rates of previously encountered individuals differ from those of first-time captures) was not significant when either grouped by sex (males: $\chi^2 = 1.07$, df =5, p = 0.96; females: $\chi^2 = 2.76$, df = 5, p = 0.74), or across the complete dataset ($\chi^2 = 1.33$, df = 5, p = 0.68). These results indicate that previously encountered snakes do not have higher rates of survival than those caught for the first time.

The results of Test 3.Sm, (whether subsequent detectability of previously encountered individuals differ from those of first-time captures) were statistically significant when running the dataset as a whole ($\chi^2 = 13.59$, df = 1, p = <0.01), as well as when the data was split into males ($\chi^2 = 7.25$, df = 1, p = <0.01), and females ($\chi^2 = 3.92$, df = 1, p = 0.048). This indicates a degree of temporal autocorrelation, in that snakes that were encountered in Year Y, were more likely to be observed in Year Y+1, than snakes first encountered in Year Y-1, but not again in Year Y.

4.4.2 Scenario 1

In this scenario, grass snakes were assumed to be unable to recover from skin lesions caused by ophidiomycosis, so remained classified as 'diseased' even if no lesions were detected on subsequent captures. The best fit model [Phi(~time + lesion)p(~time + lesion)] was used to determine the estimates of survival and detectability (Table 4.5). The range of estimated annual survival in snakes without skin lesions varied between 0.276-0.778, with a mean of 0.639 (95% CI = 0.495-0.783; Fig. 4.1a). In snakes with skin lesions, these annual estimates ranged from 0.049-0.320, with a mean of 0.224 (95% CI = 0.146-0.302). These estimates are significantly different to one another, as there is no overlap in the confidence intervals.

Table 4.5: The AIC table for Scenario 1, indicating the variables included in each model. Phi = annual survival; p = annual detectability.

Model	**Parameters**	**AIC**	**ΔAIC**	**Weight**	**Deviance**
Phi(~time + lesion)p(~time + lesion)	14	819.55	0.00	0.57	791.08
Phi(~time)p(~time + lesion)	13	820.16	0.60	0.42	793.75
Phi(~lesion)p(~time + lesion)	8	827.42	8.07	0.01	809.42

Detectability estimates differed significantly between snakes without lesions and those exhibiting them. The range of estimated detectability between years in snakes without skin lesions was 0.021-0.167, with a mean of 0.086 (95% CI = 0.04-0.134), whilst that for snakes with skin lesions was higher, ranging from 0.478-0.893, with a mean of 0.721 (95% CI = 0.595-0.848 Fig. 4.1b).

4.4.3 Scenario 2

As with Scenario 1, the best fitting model was [Phi(~time + lesion)p(~time + lesion)], from which the estimates of survival and detectability are displayed below (Table 4.6). The range of estimated annual survival in snakes without skin lesions was 0.21-0.75, with a mean of 0.53 (95% CI = 0.37-0.69; Fig. 4.1c). In snakes with skin lesions, these estimates ranged from 0.06-0.43, with a mean of 0.23 (95% CI = 0.15-0.36).

Table 4.6: The AIC table for Scenario 2 indicating the factors explored in each model. Phi = annual survival; p = annual detectability.

Model	Parameters	AIC	ΔAIC	Weight	Deviance
Phi(~time + lesion)p(~time + lesion)	14	820.22	0.00	0.78	792.22
Phi(~time)p(~time + lesion)	13	823.08	2.86	0.19	797.08
Phi(~lesion)p(~time + lesion)	9	826.80	6.58	0.03	808.80

As with the previous scenario, the model indicated a greater likelihood of detection for snakes with skin lesions, compared to those without (Fig. 4.1d). The range of estimated detectability in snakes without skin lesions was 0.03-0.32, with a mean of 0.159 (95% CI = 0.07-0.25); this was higher in snakes with skin lesions, which ranged from 0.425-0.91, with a mean of 0.69 (95% CI = 0.53-0.86).

4.4.4 Survival and detection probability modelling conclusions

Assuming that grass snakes can change states between being infected or not by *O. ophidiicola*, leads to similar results compared to Scenario 1 (Fig. 4.1); this result is not unexpected given the relatively small number of state changes outlined in Table 4.3. Both modelling approaches indicate that the presence of skin lesions (as a proxy for ophidiomycosis) decreases the apparent annual survival of *N. helvetica*, while also increasing the infected snakes’ detectability.

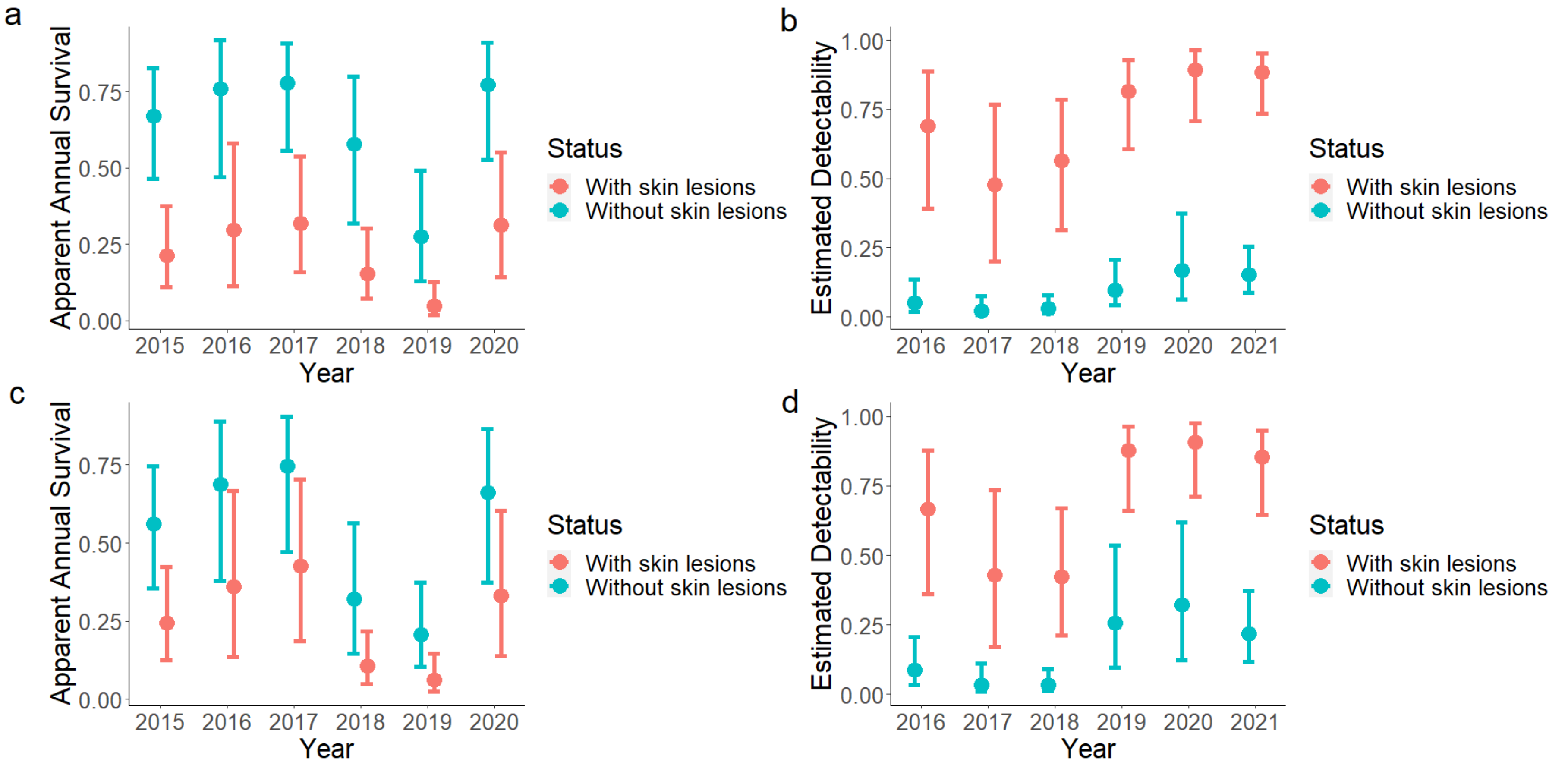


Figure 4.1: Annual survival estimates (dots) as a probability for barred grass snakes (*Natrix helvetica*) with and without detected skin lesions captured from 2015 to 2020, and estimates of detectability for *N. helvetica* captured from 2016 to 2021. Also shown are the lower and upper confidence limits (bars). The estimates of apparent annual survival and detectability for Scenario 1, assuming that skin lesions are permanent, are shown along the top row (a & b), with the estimates for Scenario 2, assuming that affected snakes may recover and clear infection, along the bottom row (c & d).

4.4.5 Population estimates

The annual population estimates of the marked sub-population (those snakes that were captured and photographed) fluctuate through time, ranging from 323 to 780 individuals (SE: 66.15-179.67; Fig. 4.2), this indicates some losses and recruitment over the study period, due to the variability in the estimate. However, this difference between yearly population estimates is non-significant when using a linear regression (F = 2.06, df = 1,5, p = 0.211). The upper estimate of the population size at 780 individuals is ~70% of the actual number of snakes encountered across the study period, based on the scale pattern identification of individuals (n = 1143). From 2019, approximately only a third of the estimated snake population was encountered per year, indicating that many went undetected across the study site. The results also indicate a non-significant trend in population size through time.

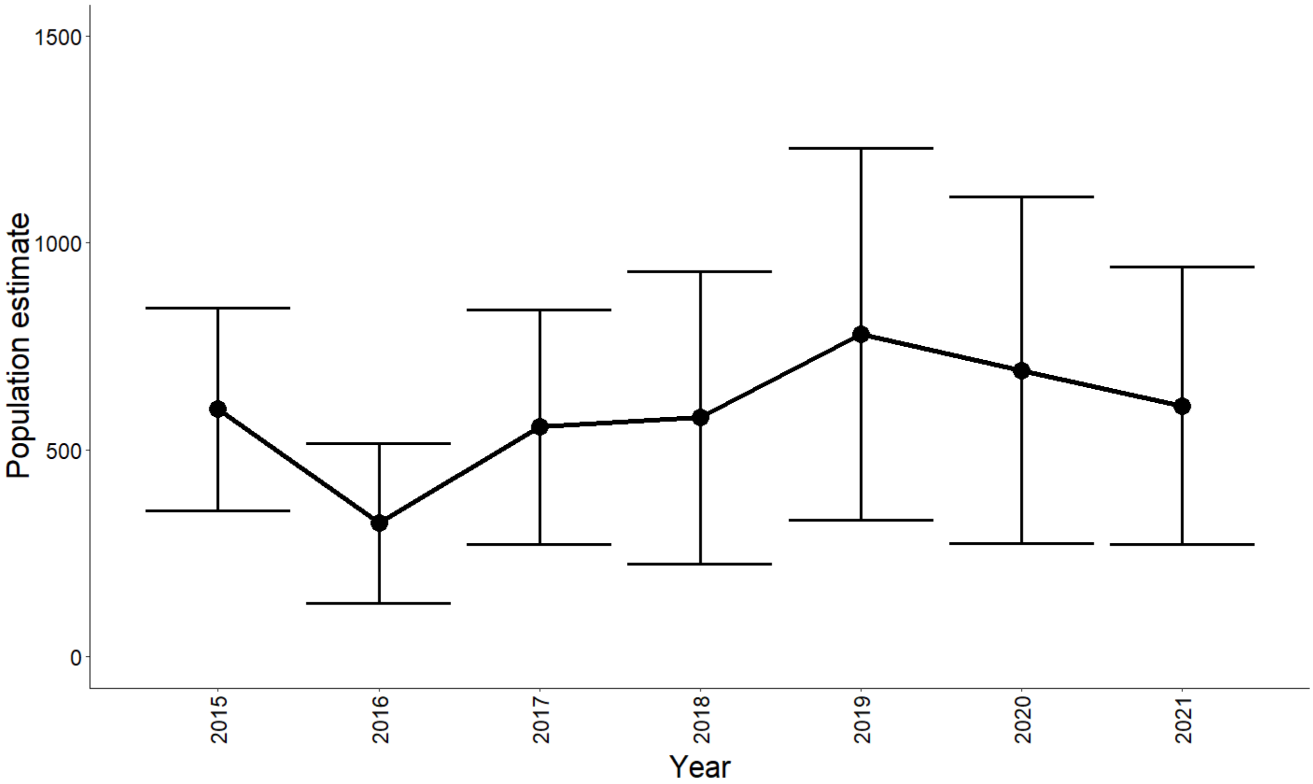


Figure 4.2: Annual population estimates of the sub-population of barred grass snakes (*Natrix helvetica*) being captured at Watermill Broad Nature Reserve, 2015 and 2021 inclusive, based on the Jolly-Seber model outputs. Bars indicate the 95% confidence intervals around each of the estimates.

4.5 Discussion

4.5.1 Annual variation

Over the seven years of data collection, there was significant annual variation in both the estimated survival and detectability of snakes. The data collected over the period of 2015 to

2018 was completed by four different MSc students, with varying levels of effort and experience, with the surveys from 2019 completed by a more experienced surveyor. The increase in estimated detection from 2019 may therefore be explained by the experience of the surveyor, although it is equally likely to be a function of the increased time spent catching snakes during the final three years.

4.5.2 Effect of skin lesions on survival

Both modelling scenarios outlined above estimated that the apparent annual survival of *N. helvetica* with skin lesions is significantly lower than for those without lesions, while the reverse is true with respect to detectability. Our mean estimates of survival (0.639 in Scenario 1, and 0.531 in Scenario 2) for snakes without skin lesions are similar to those of other studies conducted in wild snake populations, including one study of *N. helvetica* at a different site in eastern England from 2004 to 2012 (0.66, 95% CI 0.543–0.755; Sewell *et al*., 2015). Lind *et al*. (2005) found that the apparent annual survival rate of Pacific coast aquatic garter snake (*Thamnophis atratus*) was 0.636 (SE: ± 0.021) for females, and 0.558 (SE: ± 0.026) for males, over a 16-year period based on three to five surveys a year, although they did not investigate the potential for transience within the population. More recently, Breininger *et al*. (2012) concluded that the estimated annual survival rate in the eastern indigo snake (*Drymarchon couperi*) varied depending on the habitat type, from ~0.5 to ~0.9.

The similarity of results relating to apparently uninfected snakes generated by this study and those in the literature builds confidence in the novel estimates generated for apparently diseased snakes. The apparent annual survival estimates for those *N. helvetica* with skin lesions presented here were almost identical for both scenarios, with 0.224 for Scenario 1, and 0.225 in Scenario 2. This demonstrates that the presence of skin lesions associated with ophidiomycosis, may reduce the annual survival of affected *N. helvetica* by between 57.6-64.9%. No moribund snakes were encountered during our study and the severity scores for those snakes with skin lesions, were generally mild to moderate, with few severe cases (see Chapter 3). Very few carcasses were found at the site, and of those that were in a suitable condition to allow post-mortem examination, had trauma or an undetermined factor diagnosed as the proximate cause of death. Fatal ophidiomycosis cannot be ruled out however, but as most skin lesions observed in living snakes were mild, the likelihood of encountering a carcass with severe lesions and viscera intact are low.

This study therefore found no evidence to indicate that ophidiomycosis in *N. helvetica* in Europe leads to the development of skin lesions as severe as those observed in North

America (Lorch *et al*., 2015), suggesting that direct mortality due to infection is unlikely to be driving reduced survival rates. It remains possible, however, that severely affected grass snakes may have experienced a reduction in mobility such that they were no longer able to utilise the ACOs and were therefore no longer encountered. A more likely mechanism mediating survival of diseased snakes might be resultant changes in behaviour.

Researchers in North America have found that infected snakes spend more time basking to thermoregulate, possibly due to the heightened energetic demands associated with the immune response, or as a behavioural response to clear infection (Tetzlaff *et al*., 2017). Snakes from North America in the same family as *N. helvetica* (Colubridae) have been shown behaviourally to induce a fever to help clear infections through increased basking (Burns *et al*., 1996). The higher occurrence of trauma of snakes with concurrent skin lesions in this study (Chapter 3) is potential evidence of changes in behaviour, which may lead to an increased probability of indirect mortality through predation of affected snakes.

In contrast to the results presented above, McKenzie *et al*. (2020) demonstrated that the between-month survival rates calculated for two species of North American snakes (*Regina septemvittata* and *Nerodia sipedon*) when infected with ophidiomycosis, was the same as that for uninfected snakes. The estimates of monthly survival were 0.99 for both species over a 4.5 month period between early June and October. We were unable to generate equivalent monthly estimates (see Supplementary Information 4.7.2) but our results suggest that impacts are expressed over longer time periods, and highlight the need for more long-term monitoring studies to assess disease impacts on the survival of snakes at the population level.

4.5.3 Effect of skin lesions on detectability

There was a significant difference in the estimated detectability of *N. helvetica* individuals with and without skin lesions. Our estimates indicate that the mean detectability for snakes without skin lesions was 0.08 in Scenario 1, and 0.163 in Scenario 2. The latter are comparable to those from previously published studies, ranging from 0.15-0.17 (Sewell *et al*., 2015; Ward *et al*., 2017), which predate the detection of *Oo* in Great Britain

Detectability estimates calculated in this study increases to a mean of 0.721 in Scenario 1 and 0.693 in Scenario 2, for those snakes with skin lesions. The increase in detectability of snakes with skin lesions may be due to an increase in basking duration and therefore, heat seeking behaviour with an increased use of ACOs, which, as discussed above, provides support for the hypothesis that indirect impacts of behavioural change are responsible for the reduction in apparent annual survival of infected snakes.

4.5.4 Future priorities

The population estimates indicate that, while there is substantial annual variability in the number of *N. helvetica* utilising Watermill Broad Nature Reserve, there is no clear temporal trend in the size of the population monitored. Given the magnitude of the disease-related survival impact observed, this could suggest that the prevalence is stable, either because some individuals may be immune to infection, recover from disease, avoid exposure, or possibly due to immigration of uninfected snakes from other sites due to the small number of neonates encountered. It is not known at this point if the productivity is sufficient to compensate for losses. Alternatively, the power to detect an impact may be limited by the relatively short data run, an issue that could potentially be resolved by continuation of monitoring. Further refinement of our modelling in the future should include multi-state methods, such as those described by Conn & Gooch (2009), and Hudson *et al*. (2016), which have a higher sensitivity for changes in disease states. While changes in disease status may not always necessarily be observed, they can be estimated using the frequency data we present. This method was not employed in this project, however, due to limitations in the quantity of data from the historic dataset, 2015-2018.

4.5.5 Conclusion

Our study of this focal *N. helvetica* population clearly demonstrates that the presence of skin lesions, as a proxy for ophidiomycosis infection, has negative effects on survival estimates, reducing rates by approximately 50%. The lack of carcass observations and the comparatively mild severity of skin lesions detected suggest that the reduced survival is driven by behavioural change, which may influence predation rates, rather than through direct impacts of disease. The interpretation of our results would be improved through a greater understanding of the degree to which slough state influences detectability of skin lesions, and in the ability of individuals to successfully clear the disease over time.

4.6 Acknowledgements

I would like to thank both Rob Robinson and Philipp Boersch-Supan from the British Trust for Ornithology for their assistance with the modelling described within this chapter, along with Rachel McCrea from the Statistical Ecology @ Kent (SE@K) research group, and Olivier Gimenez from the Centre d'Ecologie Fonctionnelle et Evolutive, Paris with their

additional help with troubleshooting the analyses within. Thanks must also be paid to the previous four Master's students and their supervisor Iain Barr, for their valiant efforts in collecting the historic survey data used.

4.7 Supplementary Information

4.7.1 RMark Input Files

As the files are over 1140 rows in length, the option has been made to host them securely on Google Drive. They can only be accessed by following the links below.

Scenario 1: RMark input file.

Scenario 2: RMark input file.

4.7.2 Preliminary modelling attempts

When first attempting the analyses, daily models were used to try to estimate the survival and detectability of snakes within each of the sample years, using data generated by the four Masters students who worked on the project from 2015 to 2018, as well as that collected from 2019 onwards. The covariates included were sex, mass, snout-to-vent length, size class, the presence of skin lesions and the interactions of these between other covariates (see Table S1). Unfortunately, these models failed to converge, most probably due to the large number of blank (i.e. zero encounter) days between each of the sample years, during the time outside of the survey season.

The encounter data were then aggregated at a weekly resolution and the analysis re-run using models containing the same suite of covariates as the daily models but the convergence issues persisted, whether years were considered in isolation or as one continuous time period. Models were therefore ultimately run at an annual resolution which provided the results highlighted in Table S2.

Table S4.1: The model parameter of time indicates the marking duration of snakes, which in this initial modelling stage were daily intervals during the first attempt, and then weekly in the second (see above). The model parameter of time indicates the marking duration of snakes, which in this initial modelling stage were daily intervals during the first attempt, and then weekly in the second (see above).

Parameter	Model	Interpretation
Annual survival (φ)	Dot	Survival of snakes is constant through the sampling period
	time	Survival of snakes varies annually
	Sex	Survival of snakes varies with sex
	Mass	Survival of snakes varies with mass
	Length	Survival of snakes varies with length
	Length + mass	Survival of snakes varies with length and mass
	Length + sex	Survival of snakes varies with length and sex
	Skin lesions	Survival of snakes varies with the presence of skin lesions
	Skin lesions + mass	Survival of snakes varies with the presence of skin lesions and mass
	Skin lesions + length	Survival of snakes varies with the presence of skin lesions and length
	Skin lesions + sex	Survival of snakes varies with the presence of skin lesions and sex
	Size class + length	Survival of snakes varies with length and size class
	Year	Survival of snakes varies with the year(s) of capture
	Mass + sex	Survival of snakes varies with mass and sex
	Skin lesions + time	Survival of snakes with skin lesions varies annually
	Year + mass	Survival of snakes varies with the year(s) of capture and mass
	Number of surveys	Survival of snakes varies with the number of surveys undertaken
Annual detectability (*p*)	time	Encounter probability of snakes varies annually
	time + sex	Encounter probability of snakes varies annually with sex
	Skin lesions + time	Encounter probability of snakes varies due to skin lesions annually
	Length + time	Encounter probability of snakes varies due to the length of snakes annually
	Year	Encounter probability of snakes varies depending on the year of survey
	Skin lesions	Encounter probability of snakes varies with skin lesions
	Length	Encounter probability of snakes varies with the length of snakes
	Mass	Encounter probability of snakes varies with the mass of snakes
	Length + mass	Encounter probability of snakes varies with the length and mass of snakes
	Number of surveys	Encounter probability of snakes varies depending on the number of surveys
	Dot	Encounter probability of snakes is constant through the sampling period

Table S4.2: The AIC table for these preliminary modelling attempts, with only the first ten results shown from the 108 possible model combinations of the parameters listed above for the weekly models. Phi = annual survival; p = annual detectability.

Model	**Parameters**	**AIC**	**ΔAIC**	**Weight**	**Deviance**
Phi(~1)p(~lesions + time)	7	560.89	0.00	0.16	546.89
Phi(~time)p(~lesions + time)	11	562.25	1.36	0.08	540.25
Phi(~sex)p(~lesions + time)	8	562.30	1.41	0.08	546.30
Phi(~1)p(~lesions)	3	562.54	1.65	0.07	556.54
Phi(~weight)p(~lesions + time)	8	562.70	1.81	0.06	546.70
Phi(~svl)p(~lesions + time)	8	562.75	1.85	0.06	546.75
Phi(~lesions)p(~lesions + time)	8	562.80	1.90	0.06	546.80
Phi(~sex)p(~lesions)	4	563.82	2.93	0.04	555.81
Phi(~weight)p(~lesions)	4	564.05	3.15	0.03	556.04
Phi(~lesions + sex)p(~lesions + time)	9	564.24	3.35	0.03	546.24

4.7.3 Goodness of Fit Results

Table S4.3: The Goodness of Fit results for Scenario 1, where df are the degrees of freedom and p is the probability. Test 2 is used to test the equal catchability assumption, while Test 3 is used for the equal survival assumption. The lack of any significant results mean that these assumptions have been met by the data.

	Chi-square	**df**	**p**
TEST2	1.7199	5	0.8864
TEST3	2.1650	12	0.9991
Total	3.8848	17	0.9996

Table S4.4: The Goodness of Fit results for Scenario 2 where df are the degrees of freedom and p is the probability. Test 2 is used to test the equal catchability assumption, while Test 3 is used for the equal survival assumption. The lack of any significant results mean that these assumptions have been met by the data.

	Chi-square	**df**	**p**
TEST2	2.0852	5	0.8372
TEST3	4.2509	12	0.9785
Total	6.3361	17	0.9906

Chapter 5. Behavioural responses to capture in the barred grass snake (*Natrix helvetica*)

5.1 Abstract

As a non-venomous snake, the barred grass snake (*Natrix helvetica*) has a repertoire of behaviours it displays when faced with the threat of predation. It is assumed that snakes are likely to respond to handling by researchers in a similar fashion to attacks by predators, with capture for mark-recapture analyses presented the opportunity to quantify three commonly utilised anti-predatory behaviours, recorded at the initial capture and again during processing, and to relate them to individual characteristics. Of hissing, death-feigning and musking, the most commonly observed response to capture was musking, which was employed by 75.2% (875/1163) of snakes when first captured. However, after initial capture and during processing, 35.2% of snakes death-feigned, while 48.9% of snakes displayed no other responses to processing. The response of snakes during capture did not differ between three different size classes, sex, or the three phases of the sloughing cycle. The presence of a food bolus had no effect on the observed anti-predatory behaviours in *N. helvetica*. Snakes with skin lesions however, showed slightly longer duration of death feigning than those without. The presence of skin lesions consistent with ophidiomycosis may alter defensive behaviour of *N. helvetica*.

5.2 Introduction

Predation is a key selective pressure of behaviour in snakes (Rodríguez-Cabrera *et al*., 2014). The typical predators of barred grass snakes (*Natrix helvetica*) include corvids and birds of prey (Smith, 1951; Appleby 1971; Madsen, 1987), as well as mammals such as the red fox (*Vulpes vulpes*) and badger (*Meles meles*) (Beebee & Griffiths, 2000). The fact that *N. helvetica* is non-venomous may increase the risk of attack, and barred grass snakes are particularly vulnerable when they are young, due to their small size and resemblance to other potential prey, such as worms (Smith, 1951; Appleby, 1971).

Most animals, including reptiles, have developed a number of behavioural strategies in order to reduce the risk of injury or death when faced by a potential predator and escape is not possible. A common, widespread and well-studied form of antipredator behaviour among vertebrates is that of death feigning, formerly called letisimulation (Fuentes *et al*., 2021) and sometimes known as thanatosis (Rogers & Simpson, 2014). For the purpose of this project, the behaviour will be referred to as death feigning. By feigning death, the animal remains immobile, thereby reducing the visual stimuli that induce attack, minimising the risk of any further short-term damage, and increasing the likelihood of an escape (Gallup *et al*., 1971; Gregory, 2008; Rogers & Simpson, 2014). Death feigning is often seen as the last resort, used only after the prey has been detected by a predator, regularly occurring shortly after physical contact between the two (Humphreys & Ruxton, 2018; Fuentes *et al*., 2021). This behaviour has been recorded in fish (Howe, 1991; Freret-Meurer *et al*., 2017), amphibians (McCallum, 1999; Gally *et al*., 2012), mammals (Francq, 1969), birds (Sargeant & Eberhardt, 1975) and reptiles (Sannolo *et al*., 2014; Patel *et al*., 2016). It also functions as a deterrent due to the reduced appeal of carrion in some predators (Toledo *et al*., 2011).

Snake species vary in the extent to which they display thanatosis when encountered by a predator. Some species remain rigid whilst rolling on their backs (Jordan, 1970; Thomas & Hendricks, 1976), whereas others do not and remain limp throughout (Mutoh, 1983; Tryon & Guese, 1984); some may also open their mouths and gape whilst on their backs (Mori & Burghardt, 2008), although this behavioural trait is not shared across all species (Doody *et al*., 1996). Tonic immobility is regarded as the behaviour where snakes remain rigid, and frequently roll over, when displaying other antipredator behaviours. Death feigning can be viewed as a variation of tonic immobility (Gregory *et al*., 2007; Humphreys & Ruxton, 2018), given the wide-range of behavioural responses described above. This behaviour is also sometimes accompanied by autohaemorrhaging from the oral cavity (Gregory *et al*., 2007).

Death feigning is employed as an anti-predator response by *N. helvetica* (Gregory *et al*., 2007) but it is not the only defensive behaviour exhibited. Many snake species possess a pair of cloacal glands, which produce a malodorous liquid (Kissner *et al*., 1998); these glands are larger in female *N. helvetica* compared with the males (Thorpe, 1989). This liquid is released when snakes are captured by a potential threat, often combined with faeces, in order to deter the predator (Beebee & Griffiths, 2000), a process known as musking. The musk may also assist in the action of death feigning, by making the snake smell and taste more like a rotting corpse, thereby mimicking carrion (Kissner *et* al., 1998). Grass snakes may also produce loud audible hisses, before mock striking with the head (Beebee & Griffiths, 2000; Golubović *et al*., 2021).

Snakes are likely to react to researchers in the same way as they would to their natural predators, creating an opportunity to quantify defensive behaviours. There is significant individual variation in the extent to which such behaviours are exhibited; Gregory *et al*.

(2007), for example, found that 66% percent of *N. helvetica* feigned death on capture. The probability of each anti-predator behaviour being displayed may be mediated by the experience of individuals, which in turn may be influenced by age. Physiology may also play a role in the likelihood of musking, which might be predicted to be significantly higher in females due to their larger cloacal glands. Variation in energetically costly behaviours may also be dependent on condition, which could be linked to age, to sex if females are investing more than males in breeding, or to the stage of the slough cycle, as replacement of the dermal layer also requires significant investment. The occurrence of disease could therefore also play a role if this influences energetic state when snakes cannot bask frequently enough, which could provide a potential mechanism for the lower apparent annual survival rates observed in snakes presenting skin lesions consistent with apparent ophidiomycosis (Chapter 4).

This study aims to explore the factors responsible for individual variation in defensive responses but there is a secondary aim. When conducting ecological surveys, researchers also have a duty to ensure the welfare of the animals involved is not compromised, and that the design of experiments minimises stress to the study organisms. The capture and manipulation of reptiles can have negative health effects, such as stress which might lead to immunosuppression (Silvestre, 2014). This is of particular interest given the recent detection of ophidiomycosis in *N. helvetica* populations (Franklinos *et al.*, 2017), and the findings presented in Chapters 3 and 4, as prolonged stress could exacerbate disease occurrence and/or severity (Dohms & Metz, 1991). The first step to reduce any associated impacts is to document the repertoire of behaviours presenting upon capture and processing. Monitoring the behaviour of individuals during handling can therefore inform the impact of the intervention on animal welfare, and be used to improve methods (Soulsbury *et al*., 2020).

5.3 Materials and methods

5.3.1 Capture methodology

The capture methodology is outlined in detail in Chapter 2. Captures took place from May to September in 2019 and 2021, and from July to September in 2020 as a consequence of the COVID-19 pandemic. Sixty-nine artificial cover objects (ACOs), placed across the site field site (Watermill Broad Nature Reserve, Cranwich, Norfolk, UK) were used to detect and capture individuals. Half of the ACOs were checked on each capture visit throughout the period, and a maximum of two individuals were caught from each ACO by hand to avoid prolonged processing times.

5.3.2 Recording snake behaviour

The defensive reaction of snakes on initial capture was recorded immediately, and the behaviour of each snake was then recorded again during the processing phase. The response of each snake was allocated to one of the following anti-predator responses:

- Feigned death (thanatosis): Snakes played dead, typically rolling onto their back, sometimes gaping, with the tongue extended, and occasionally autohaemorrhaging (Fig. 5.1).
- Hissing: Snakes made an audible hiss.
- Musking: Secretion of a foul-smelling anti-predator fluid from the snake's anal glands, sometimes mixed with faeces.
- No observed change: Snakes displayed none of the aforementioned behaviours.

Figure 5.1: The barred grass snake (*Natrix helvetica*) feigning death, one of the antipredator responses most frequently observed during this study. Note that the snake has rust-coloured skin lesions that are consistent with ophidiomycosis on the ventrum (indicated with an arrow).

Only a very small number of snakes (n = 12) demonstrated more than one of the three listed antipredator behaviours during either the initial capture, or during processing. Consequently the decision was made to include the first behaviour exhibited in the analyses, such that only one response was recorded for each snake during the initial capture period, and a second response was recorded during processing. Only two snakes across the three years of data collection were released immediately after capture due to excessive thrashing and writhing, which is a potential indication of severe stress.

Immediately after capture, and once initial defensive responses had been recorded, snakes were placed in polyethylene bags and then in a black cloth bag to provide a dark environment that reduced stress following the initial capture. The initial capture event frequently lasted only a few seconds but snakes captured alone were left in the bag for 30-45 seconds before processing. Subsequent handling and data collection took circa 3-4 minutes so, if two snakes were caught simultaneously, the second snake spent approximately five minutes in the bag prior to processing. Each individual was examined for the presence/absence of skin lesions consistent with ophidiomycosis, sexed, measured, and the slough cycle was evaluated, following the methods outlined in Chapter 2.

Each capture occasion was also classified as either ‘new’, if the snake had not been captured previously during the season in question, or ‘recapture’, if it had; a binary variable was included in models rather than a continuous variable detailing encounter number due to the long tail in the distribution of recaptures. These data would help to provide evidence for either habituation or sensitisation to capture and processing.

5.3.3 Modelling approaches

Generalised Linear Mixed Models (GLMMs) were used to explore the association of sex, size class, presence of skin lesions, presence of a food bolus, stage of the slough cycle and capture occasion all included as categorical variables, on each of the three primary defensive responses (i.e. death feigning, hissing and musking), during the initial capture only. The presence/absence of each behavioural response was modelled using a separate GLMM, specifying binomial errors and a logit link function, with individual snake identity included as a random factor to correct for pseudoreplication within the dataset. A further GLMM of identical structure was used to model the influence of these explanatory variables on the presence/absence of any type of defensive response, with death feigning, hissing or musking recorded as ‘1’ and the absence of any of these behaviours recorded as ‘0’. This was completed to determine whether or not any factor was associated with the observations of anti-predator behaviours as a whole. Finally, the generalized variance-inflation factor (GVIF) was used to test whether or not there was a correlation between any of the covariates used in the GLMMs factors (Fox & Monette, 1992).

5.3.4 Recording duration of death feigning and statistical analysis

During 2021, when snakes were observed to be death feigning upon capture or during processing, the duration at which this behaviour lasted was recorded (to the nearest second) when it lasted for at least five seconds. This was to ensure that snakes were truly feigning death, and not one of the other variants of tonic immobility. An ANOVA was then used to determine the association between the presence of skin lesions, sex, size class, presence of a food bolus and sloughing on the duration of death feigning, in *N. helvetica*.

5.4 Results

Over the study period (2019-2021), 1164 snake capture events were recorded involving 649 individuals. The tables below summarise captures by sex (Table 5.1), size class (Table 5.2), and number of recaptures (Table 5.3), displaying data from all captures. After removing neonates from the dataset due to the small sample size, and those captures where the sex of snakes could not be determined, a total of 1100 observations were used in the GLMMs.

Table 5.1: Summary of the number of barred grass snakes (*Natrix helvetica*) captured by sex; seven of the snakes found in 2020 and one of the snakes from 2021 were of undetermined sex.

	Female	**Male**
2019	201	236
2020	172	178
2021	173	197

Table 5.2: Summary of the number of barred grass snakes (*Natrix helvetica*) captured by size class. Seven of the snakes found in 2020 and one of the snakes from 2021 were of undetermined size class.

	Neonate	**Sub-adult**	**Adult**
2019	7	228	202
2020	6	216	128
2021	17	195	158

Table 5.3: Summary of the distribution of within-season recaptures of the barred grass snake (*Natrix helvetica*).

Year	**Number of encounters**	Avg. time between recaptures (days)

	1	**2**	**3**	**4**	**5**	**6**	**7**	
2019	294	86	38	12	3	2	2	20.22 (1 – 123)
2020	244	72	24	10	4	3	0	7.55 (1 – 58)
2021	214	80	40	22	9	4	1	15.79 (1 – 93)

5.4.1 Comparison of responses during the initial capture and processing phases

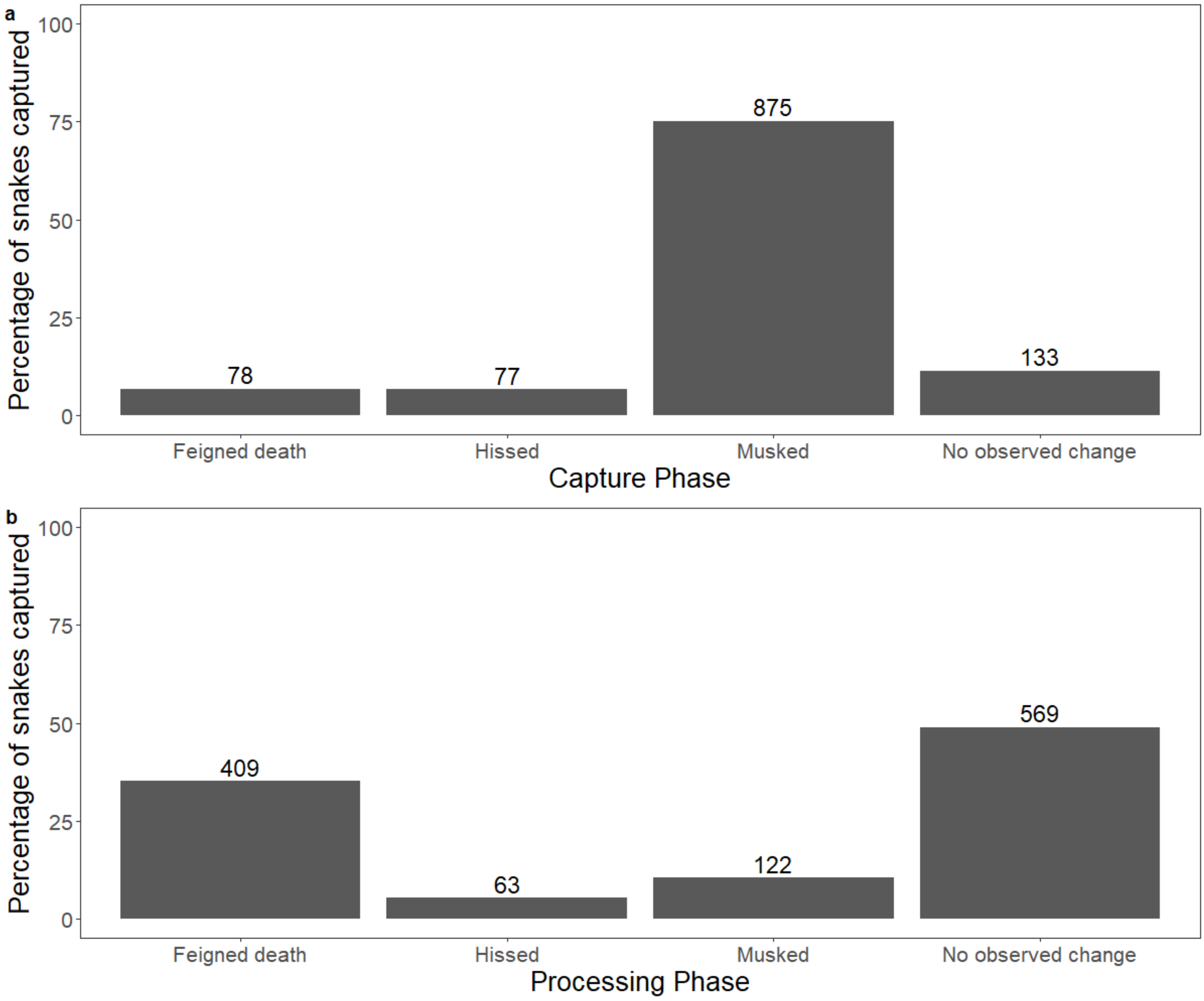


Figure 5.2: The percentage of individual barred grass snakes (*Natrix helvetica*) displaying each of the four observed antipredator behaviours during (a) the initial capture stage; and (b) the processing stage. The data displayed above the bars indicates frequency and includes

every capture instance, including recaptures. Two instances of food regurgitation have been removed from the recorded observation shown in the graph.

Two snakes were observed regurgitating food but this sample was too small to permit inclusion of this behaviour in the analysis. Snakes performed other antipredator behaviours in addition to those listed above, but again the infrequent occurrence of mock striking (n = 4) and hooding (n = 6), which prevented analysis.

A Chi-square test comparing the anti-predator responses observed at initial capture with those observed during processing, including only the first encounter with each individual snake, indicated that there was a was statistically significant difference, $\chi^2 = 568.7$, df = 3, p = <0.0001. Musking was the most frequently recorded response at the initial capture, whereas death feigning, or the absence of any defensive response, was recorded most frequently during processing (Fig. 5.2).

5.4.2 Influence of individual characteristics on defensive response

The generalised variation inflation factor (GVIF) indicated that there was no correlation between any of the factors explored as part of the GLMMs used within the analysis described below (Table 5.4). There were no significant correlations between the covariates, as the scores provided were between 1 and 2 (Fox & Monette, 1992).

Table 5.4: The generalised variation inflation factor (GVIF) for each of the covariates used within the GLMMs outlined in Section 5.3.3, including the degrees of freedom for each too.

Factor	GVIF	df
Food	1.02	1
Sex	1.02	1
Size class	1.10	1
Skin lesions	1.16	1
Slough cycle	1.14	2
Capture occasion	1.06	1

The results of the GLMMs exploring factors influencing the occurrence of death feigning during the initial capture indicated that those snakes that had been captured previously were less likely to display this behaviour (Table 5.5). This may indicate some degree of habituation to capture, following recapture in *N. helvetica*.

Table 5.5: Parameter estimates, standard errors, z-values, and p-values for the generalised linear mixed model (Food + Sex + Size Class + Skin Lesions + Slough Cycle + Capture Occasion), with the frequency of death feigning during the capture phase as the dependent variable. Significant values are marked with an asterisk.

Parameter	Estimate	Standard error	z-value	p-value
(Intercept)	-8.75	1.58	-5.55	<0.01
Sex	0.19	0.93	0.19	0.84
Size class	0.34	1.02	0.34	0.74
Skin lesions	0.89	1.10	0.81	0.42
Slough cycle: Phase 1	0.14	0.71	0.19	0.85
Slough cycle: Phase 2	-7.16	3.75	-1.91	0.06
Capture occasion: Recaptures	-1.54	0.60	-2.56	0.01*
Presence of food	-0.12	0.66	-0.18	0.85

None of the potential explanatory variables demonstrated a statistically significant relationship with the tendency of snakes to hiss in response to initial capture (Table 5.6). The presence of a food bolus was excluded from the analysis due to unexplained interactions, which led to all explored factors as being significant.

Table 5.6: Parameter estimates, standard errors, z-values, and p-values for the generalised linear mixed model (Sex + Size Class + Skin Lesions + Slough Cycle + Capture Occasion), with the frequency of hissing during the capture phase as the dependent variable.

Parameter	Estimate	Standard error	z-value	p-value
(Intercept)	-9.27	1.27	-7.29	<0.01
Sex	0.81	0.78	1.03	0.30
Size class	0.23	0.82	0.28	0.78
Skin lesions	0.72	0.72	0.99	0.32
Slough cycle: Phase 1	-0.06	0.59	-0.09	0.92
Slough cycle: Phase 2	0.46	0.73	0.63	0.53
Capture occasion: Recaptures	-0.58	0.48	-1.21	0.22

None of the potential explanatory variables demonstrated a statistically significant relationship with the tendency of snakes to musk in response to initial capture (Table 5.7).

Table 5.7: Parameter estimates, standard errors, z-values, and p-values for the generalised linear mixed model (Food + Sex + Size Class + Skin Lesions + Slough Cycle + Capture Occasion), with the frequency of musking during the capture phase as the dependent variable.

Parameter	Estimate	Standard error	z-value	p-value
(Intercept)	1.20	0.21	5.69	<0.01
Sex	-0.02	0.16	-0.17	0.87
Size class	-0.09	0.17	-0.49	0.62
Skin lesions	-0.33	0.19	-1.66	0.09
Slough cycle: Phase 1	-0.11	0.19	-0.60	0.55
Slough cycle: Phase 2	0.11	0.23	0.47	0.64
Capture occasion: Recaptures	0.27	0.16	1.72	0.09
Presence of food	0.29	0.16	1.92	>0.05

No statistically significant relationship was observed between the tendency to exhibit a defensive response of any type at initial capture and any of the explanatory variables (Table 5.8).

Table 5.8: Parameter estimates, standard errors, z-values, and p-values for the generalised linear mixed model (Food + Sex + Size Class + Skin Lesions + Slough Cycle + Capture Occasion), with the frequency of any behavioural response during the capture phase as the dependent variable.

Parameter	Estimate	Standard error	z-value	p-value
(Intercept)	7.96	0.81	9.79	<0.01
Sex	-0.11	0.48	-0.24	0.81
Size class	-0.95	0.55	-1.74	0.08
Skin lesions	-0.12	0.55	-0.21	0.83
Slough cycle: Phase 1	-0.05	0.49	-0.10	0.92
Slough cycle: Phase 2	0.21	0.57	0.36	0.72
Capture occasion: Recaptures	0.61	0.38	1.58	0.11
Presence of food	0.69	0.39	1.77	0.08

5.4.3 Duration of death feigning during the processing phase

A total of 94 out of 409 observations of death feigning lasting for periods longer than five seconds were recorded, all of which occurred during the processing phase, were included in the analysis described below. Only four periods with a duration >5 seconds were recorded during the capture phase, from 78 capture events. Therefore, due to the small sample size, these were excluded from any analysis, and the duration of death feigning focussed solely on the processing phase.

Those snakes with skin lesions feigned death for significantly longer than those without, F(1,91) = 4.52, p = 0.04 (Fig. 5.3a). There were no differences observed in the duration of death-feigning when comparing the two sexes, F(1,91) = 0.16, p = 0.69 (Fig. 5.3b), the presence or absence of a food bolus, F(1,91) = 0.09, p = 0.77 (Fig. 5.3d), or when comparing the three stages of the slough cycle, F(1,91) = 0.22, p = 0.81 (Fig. 5.3e). Adults however, consistently feigned death for longer periods of time than sub-adults, F(1,91) = 4.30, p = 0.04 (Fig. 5.3c).

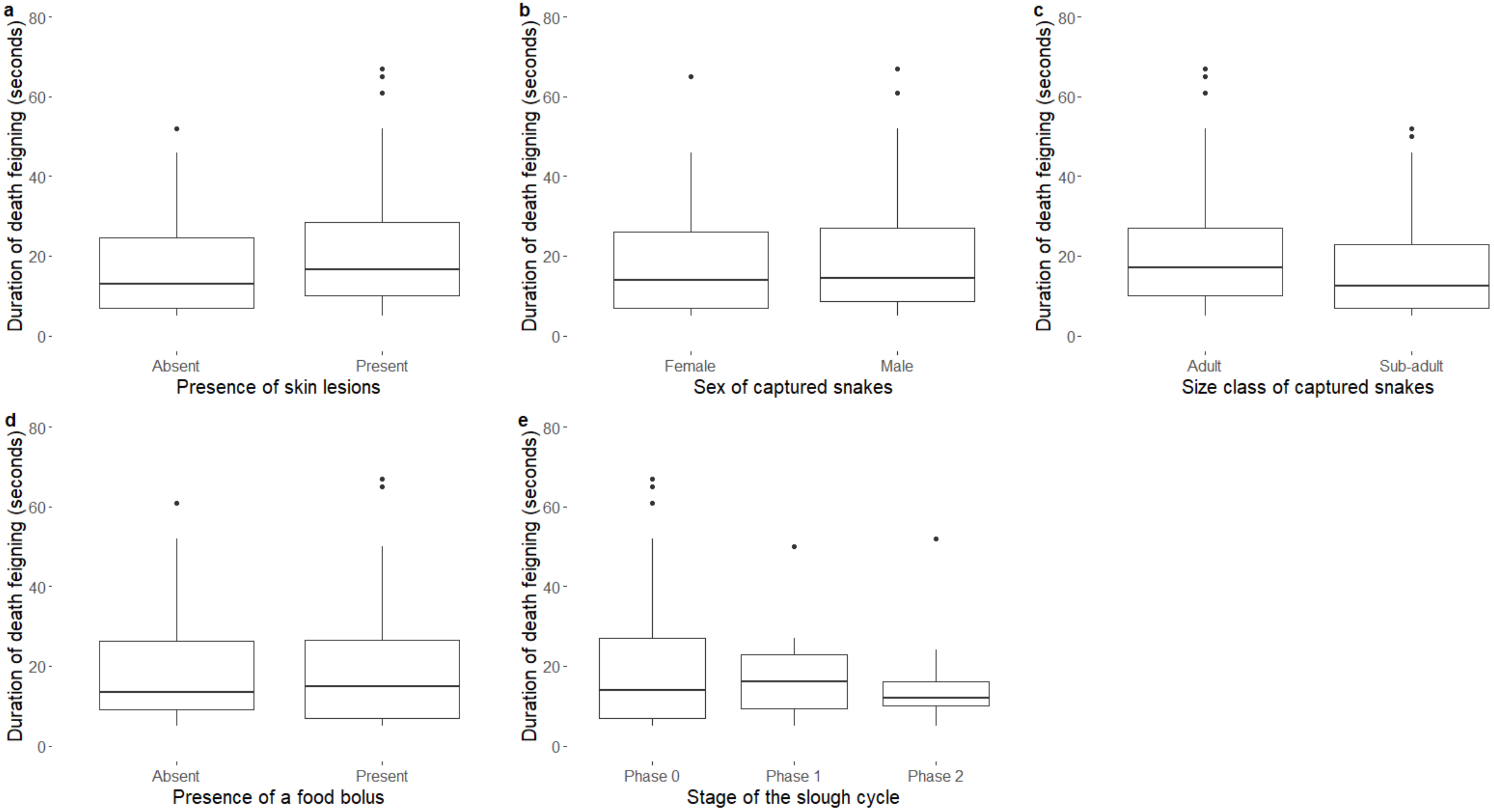


Figure 5.3: Boxplots indicating the time snakes spent death feigning during the processing phase, for each of the categories described above: presence of skin lesions (a), sex (b), size class (c), presence of a food bolus (d), and the slough cycle (e). Plotted are the medians, interquartile ranges (indicated by the boxes), ranges (lines), and outliers (black dots).

5.5 Discussion

While previous studies have documented the occurrence of death feigning (Gregory *et al.*, 2007), this is the first to relate the frequency at which a suite of anti-predatory behaviours was observed in a wild population of *N. helvetica* to individual characteristics such as size, sex and disease status. Eighty–eight percent of barred grass snakes demonstrated anti-predator behavioural responses upon the initial capture but this figure fell to 51.0% during the subsequent processing phase. During initial capture, musking was most commonly observed, displayed by 75.2% of individuals, while death feigning was relatively rare, recorded in just 6.6% of snakes. In contrast, during processing, only 10.5% of snakes musked, while 35.2% feigned death. The lower occurrence of defensive behaviours exhibited during processing may be because death feigning is used as a last resort (Beconi *et al.*, 2019), whereas musking is most effective upon initially being captured, but is only of limited use if this does not deter the predator.

There was limited evidence that apparent disease status influenced defensive behaviours observed in *N. helvetica*. There was no relationship in the occurrence of death feigning, hissing, or musking recorded in those snakes with skin lesions, compared to those snakes without. The only significant change observed was that snakes with skin lesions feigned death for significantly longer, than those without. This may indicate ways that ophidiomycosis may alter the behaviour of *N. helvetica*. These differences may be apparent because of changes in the thermoregulatory behaviour of snakes (Lorch *et al.*, 2015; Tetzlaff *et al.*, 2017) caused by ophidiomycosis, which may mean they need to spend more time basking than usual. Death feigning may also be a less successful approach to predation avoidance, compared to the other potential behaviours that snakes could opt for, where conflict is evaded completely. This could therefore potentially contribute to the reduced survival rate observed in those snakes with skin lesions (see Chapter 4).

There were no significant differences in the behavioural responses to a potential predator between adults and sub-adults. This is consistent with the findings of Gregory *et al.* (2007) who found no variability in death feigning when accounting for body size. There were however marginally significant differences between the duration of death feigning between the two size classes, with adults feigning for longer than sub-adults. This may be a consequence of a greater number of previous encounters with predators. No differences between the responses of *N. helvetica* to capture between males and females were found. This result is similar to that seen in the closely related dice snake (*N. tessellata*), where death-feigning frequencies were the same between sexes (Golubović *et al*., 2021). Additionally, the models indicated no significant differences in the behaviour of captured *N. helvetica* when comparing the three different phases on the slough cycle, nor were differences seen in the behaviour of captured snakes caused by the presence of a food bolus.

The recapture of snakes had a significant effect on the behavioural response of *N. helvetica*, in response to a potential predator. Fewer recaptured snakes feigned death compared to those captured for the first time, indicating the potential for habituation. This may however be an artefact of a small sample size, as only 78 observations of death feigning were recorded in the initial capture phase. This result of habituation is similar to that previously observed in other *N. helvetica* populations (Gregory, 2008).

The next steps following on from this research would be to relate the capture of *N. helvetica* to an independent measure of stress such as hormones, an example being glucocorticoids that can be analysed by non-invasive faecal sampling (Augustine *et al*., 2022). Additionally, different post-capture treatments could be used (other than snakes being placed in a dark cloth bag) to determine if the behaviour of *N. helvetica* (or other closely related European colubrids) changes during processing.

5.6 Acknowledgements

We would like to thank Pat Gregory from the University of Victoria, Canada, for his input and guidance with collecting the necessary data for this chapter.

Chapter 6. General discussion

Snakes as a group have a paucity of data regarding their conservation status due to their cryptic nature, and low detection probabilities (Steen, 2010; Durso *et al*., 2011; Durso & Seigel, 2015). Understanding more about the population dynamics and identifying the threats/drivers of a snake species informs the design of conservation measures, which can reduce the risk of extinction of threatened species (Reading *et al*., 2010). While habitat loss is the biggest cause of biodiversity declines globally, the emergence of novel diseases can also lead to declines of affected species (Fisher *et al*., 2020).

This thesis explores the population dynamics of the barred grass snake (*Natrix helvetica*) at a site in Eastern England, quantifying the occurrence and potential impacts of the emerging infectious disease ophidiomycosis on individual snake survival. The study began with validation of the data collected throughout the 7-year lifetime of the project, ensuring that data were appropriate for combined analyses (Chapter 2), then moved to descriptions of skin lesions and rtPCR analyses of skin swabs for *Ophidiomyces ophidiicola* (*Oo*) DNA (Chapter 3), Cormack-Jolly-Seber models were then used to determine whether the presence of skin lesions consistent with ophidiomycosis had any effect on apparent annual survival or detectability (Chapter 4). Finally, given apparent reduction in survival rates of diseased snakes identified, the defensive behaviour of *N. helvetica* was studied to determine if ophidiomycosis may have caused detectable changes in anti-predator behaviours (Chapter 5), and to aid in minimising the impacts of handling on snake welfare in the future.

6.1 National monitoring of barred grass snake (*Natrix helvetica*) populations using capture visits and count surveys

There is a paucity of structured monitoring data for amphibians and reptiles in Great Britain, when compared to other taxa such as birds or butterflies (Pocock *et al*., 2015). Apart from this study, and others at a limited number of other sites (such as Sewell *et al.*, 2015), most of the data currently used to inform the conservation status of the barred grass snake have come from opportunistic sightings gathered by various recording schemes, or ecological surveys carried out prior to development work. These data are often shared with local Biological Records Centres, and the National Biodiversity Network (Pocock *et al*., 2015). Moreover, not all data (or associated metadata) are freely available, which means that national-scale analyses are fraught with assumptions concerning potential data gaps (Isaac & Pocock, 2015). With such a disjunct level of current data (Turner *et al*., 2022), the outcomes of this study and the survey methods described within (Chapter 2), could help inform the design of other monitoring schemes for *N. helvetica* populations in Great Britain, similar to the Make The Adder Count (Gardner *et al*., 2019).

One of the major challenges to conducting large-scale surveys in grass snakes would be the need for sufficient interest from bodies such as the Joint Nature Conservancy Committee (JNCC) or Department for Environment Food and Rural Affairs (DEFRA), and non-governmental organisations for instance the Wildlife Trusts, to provide the opportunities for funding and support, given other conservation priorities. The Wildlife Trusts are a perfect candidate for such a national scale project due to their vast network of nature reserves, which are often surveyed and monitored by both volunteers and staff.

6.1.1 Capture surveys and barred grass snakes

We have demonstrated the ability of capture surveys to produce varied data regarding different aspects of the ecology of a free-living snake species, and the occurrence of ophidiomycosis. Therefore, regional groups such as those within the Wildlife Trusts or Amphibian and Reptile Groups of the UK (ARG UK), could conduct such structured surveys in conjunction with other partners, using artificial cover objects (ACOs) placed within a small number of key sites in each county, following the methods described in Chapter 2. Such study sites may be a mix of natural or artificial wetlands (such as Watermill Broad Nature Reserve), and other suitable locations where *N. helvetica* inhabit. There are a growing number of former quarry sites that have been restored into wetlands, such as Needingworth in Cambridgeshire, that may be appropriate (Benstead, 2000). These surveys could also be conducted in conjunction or collaboration with appropriate institutions, such as local universities.

The data generated by these surveys could then be shared with the national coordinating body for the project, such as ARG UK, in order for analysis of long-term population trends to be completed on a much larger scale (Turner *et al*., 2022), than the remit covered by the current study. This would assist in identifying which of the currently recognised threats (see Foster *et al*., 2021) are the most significant throughout Great Britain, and where the development of mitigation guidance should be targeted. It is probable that different populations of *N. helvetica* experience different threats, throughout the species' range nationally. There is a growing need to increase the monitoring of *N. helvetica* on the conservation priority agenda within Great Britain, so that populations do not reach localised extinction, without our knowledge.

6.1.2 The use of count surveys to monitor populations of barred grass snakes

The aforementioned Make The Adder Count is a citizen science project run by ARG UK, that specifically targets known European adder (*Vipera berus*) overwintering sites, with surveyors visiting a repeatable transect up to a recommended six times between February and May, to count basking snakes. The ACOs used to increase the detection of *N. helvetica* could also be used to undertake count surveys of individuals, in order to try to gather some baseline population data at a number of representative sites across the country, in a similar way to the Make The Adder Count.

A count survey approach would be far less intensive that the capture methods outlined in Chapter 2. This study may help to provide and inform a framework around which a national monitoring protocol could be established, if appropriate sites where cover objects can be laid are identified. Across the three years of data collection as part of this PhD, only 41 captures were of snakes encountered outside of ACOs, indicating a high usage rate by *N. helvetica*. Barred grass snakes can be cryptic, and extremely hard to find when ACOs are not used (Ward *et al*., 2017). Count surveys were piloted as part of the long-term monitoring of *N. helvetica* at Watermill Broad Nature Reserve, the next step is to compare these data with the population estimate trend generated from the survival data. This will help to inform the study design including the frequency and phenology of such surveys, in order to produce robust population trends. These can then be used to inform the guidance accompanying the newly launched National Reptile Survey (ARC Trust, 2021).

Barred grass snakes may have increased contact rates under ACOs, but since they also congregate at other sites in the environment, such as when seeking out mates (Beebee & Griffiths, 2000), the additional risk of exposure to pathogens posed by the use of ACOs is considered likely to be low. However, there is the need to undertake studies to explore the role of ACOs in the transmission of diseases (such as ophidiomycosis) between individuals, through sampling of cover objects and comparing these with natural habitats.

6.1.3 Opportunistic collection of non-invasive samples

The collection of voided faeces from *N. helvetica* (which is a frequently observed anti-predator behaviour as part of musking, see Chapter 5) could be used for further health screening. Such samples were collected as part of the project outlined within this thesis for microbiological examination and *Salmonella* spp. isolates generated are awaiting further characterisation. Metabarcoding could also be to investigate the presence and range of endoparasites present in *N. helvetica*, from the DNA contained within the collected faecal matter (Bourret *et al*., 2021). Another potential use of the voided faeces would be to conduct dietary studies of individuals (again through the use of metabarcoding), comparing the different prey items/groups consumed by the different size classes or sex of captured snakes (Brown *et al*., 2014). The long-term study at Watermill Broad Nature Reserve was originally set up to quantify the seasonal/annual variation in the abundance of *N. helvetica*, and relate it to variation in the rates of nest predation of the Amber-listed reed bunting (*Emberiza schoeniclus*), following anecdotal evidence of snakes being a significant predator.

Other important data can also be collected opportunistically when undertaking surveys for *N. helvetica*. Snakes often leave sloughed skin remnants underneath ACOs, or in the environment so they can be located by surveyors if snake trackways are known. These can be collected, to allow for the extraction and sequencing of DNA (Jones *et al*., 2008). These data can then be used for population genetics studies to establish the levels of gene flow both within and between populations of *N. helvetica*, also identifying levels of inbreeding (Khedkar *et al*., 2016). It is important to store sloughs appropriately to allow for such analysis, without the degradation of host DNA. In the long term, this requires the sloughs to be dried before being stored in a freezer at -20˚C, in an envelope or zip-lock bag. The details of the site, species, and date should also be transferred to whichever storage container is preferred.

The collected sloughs could then be submitted to the ARC Trust Reptile Genebank Project (https://www.arc-trust.org/genebank), to help inform the population-level genetics of *N. helvetica* across the country. If further active surveys for *N. helvetica* were conducted, this would also provide the opportunity to enhance opportunistic sample collection for existing schemes, for example snake carcass submission to the Garden Wildlife Health project (https://www.gardenwildlifehealth.org/), for post-mortem examination. Sloughs collected as part of this research project have contributed to both of the aforementioned citizen science projects (Franklinos *et al*., 2017).

6.1.4 Considerations when undertaking the translocation of snakes

The translocation of *N. helvetica* (and other reptile species) is a common practice in the United Kingdom, designed to prevent harm to a species or population, during the development of a site for housing or (Nash *et al*., 2020). Reptiles are often captured *en masse*, and moved to a nearby site with a similar habitat structure as the one they have been removed from, although this is not always the case as they may be moved further away or to less suitable sites (Nash *et al*., 2020). Mitigation translocations are ‘supply’ driven, meaning they are initiated by the need to remove animals out of harm’s way, and usually do not fall within the IUCN guidelines for translocations (Germano *et al*., 2015).

This movement of snakes could lead to the spread of diseases and parasites, such as ophidiomycosis, to naïve populations, while also increasing opportunities for pathogen transmission among captured snakes (if housed together in the same temporary holding facilities). It is therefore important to develop guidelines around the potential disease risks and hazards in reptiles, and share these among ecological practitioners, disseminating the information via organisations such as Chartered Institute of Ecology and Environmental Management (CIEEM). Biosecurity guidelines already exist for amphibians (ARG UK, 2017), and should be updated to include reptiles, or an additional guidance note could be produced for reptiles, including the skin lesion severity scoring system described in Chapter 3, to allow practitioners to quickly assess the diseased barred grass snakes they encounter. An additional way to reduce the risk of spreading diseases between populations, would be to undertake disease risk analyses to inform translocations before they are undertaken (Bobadilla Suarez *et al*., 2017), following the methods proposed by Hartley & Sainsbury (2017).

6.2 Investigating the occurrence of ophidiomycosis in other *Natrix helvetica* populations

The results presented within Chapter 3 reflect the presentation and occurrence of ophidiomycosis in a single population of *N. helvetica*, although the clinical signs are likely consistent throughout the species’ range. There is the need for studying further populations of *N. helvetica* to establish the distribution of ophidiomycosis throughout Great Britain, and if appropriate to document the clinical signs and apparent prevalence of skin lesions, to allow for comparisons to be made between the results presented in Chapter 3. One such site is 30 km away from Watermill Broad Nature Reserve which has not yet been surveyed for the presence of ophidiomycosis, but does have abundance trends for *N. helvetica*. Sites such as this one could be the ideal candidate to determine if the prevalence of skin lesions observed and reduction in survival rates estimated in this study, are representative through Great Britain.

Current studies of wild snakes across multiple countries in mainland Europe are underway to learn more about the distribution, and species affected by *Oo* infection, with a skew towards semi-aquatic species (Blanvillain *et al*., 2022). This is especially important as we lack data from Great Britain pertaining to the susceptibility of *V. berus* and the smooth snake (*Coronella austriaca*). To date, only a single European adder has been reported to have tested rtPCR-positive for *Oo* DNA, although the individual lacked skin lesions (Franklinos *et al*., 2017). As European adders and smooth snakes are species of conservation concern in Great Britain and elsewhere in Europe (Reading *et al.*, 2010; Gardner *et al*., 2019), it is therefore important to establish whether or not these two species are also affected by ophidiomycosis.

If appropriate permissions are obtained, then taking scale clippings from those regions (ventral scales) of snakes with skin lesions may be a better alternative to collecting skin swabs in yielding *Oo* DNA (Maigret, 2019). This would allow for further mycological and histopathological work to be completed, as samples could also be used to culture *Oo*. As approximately 20% of the skin lesions tested were rtPCR-negative for *Oo* DNA, these scale clips would also be able to provide more *Oo* DNA (if present) than a superficial skin swab, especially if the fungal hyphae had penetrated deeper into the dermis than what could be sampled with swabs. Combining the PCR results and histopathology of these scale clips would help to determine the true false negative rate for *Oo* detection, and the aetiology of the skin lesions. They may also help in the further detection and identification of aetiological agents responsible for diseases with similar clinical signs to ophidiomycosis.

6.2.1 Environmental pathogen surveillance

Another potential line of enquiry is the role of the environment in the transmission of ophidiomycosis between individual snakes. The use of ACOs is required in the monitoring of *N. helvetica* to both standardise effort, and because data from visual encounter surveys would be very limited in comparison. No study to date has yet investigated whether or not *Oo* can survive on the surface of ACOs for periods of time. Some studies have indicated that *Oo* can persist as an environmental saprophyte (Campbell *et al*., 2021).

The current approach of disease surveillance relies on the capture of live snakes, or the discovery of dead ones, for inspection of skin lesions and subsequent swabbing. As discussed above, this is extremely time intensive and is not always applicable, especially in areas where *N. helvetica* are at a lower population density than at Watermill Broad Nature

Reserve. It may therefore be easier and more effective to survey for *Oo* itself by collecting environmental samples. Samples collected from known snake dens and the topsoil surrounding them have successfully yielded positive rtPCR *Oo* results, as well as cultures (Campbell *et al*., 2021). There is the need to develop quantitative PCR protocols and look at fungal loads on ACOs, and other areas of the environment utilised by snakes, as these may be sites of transmission for *Oo* between individuals. These results would also confirm the presence or absence of *Oo* at site, and its distribution within different habitats.

Depending on the structure of the habitat sampled, other environmental samples from the terrestrial landscape may be collected such as leaf litter and rotting wood, for the analysis of *Oo* DNA (Lopes *et al*., 2021). The use of environmental DNA (eDNA) could also potentially be used to detect *Oo* in water samples collected from sites where affected snakes have been identified (Baker *et al*., 2020), although the effectiveness of this technique has yet to be evaluated. The use of metabarcoding of environmental samples in this way provides the opportunity to identify a wide range of different microorganisms in a cost-effective manner (Abdelfattah *et al*., 2018; Francioli *et al*., 2021).

6.2.2 Investigating the global distribution and diversity of *Oo* lineages

6.2.2.1 The use of museum collections

There is still the question of whether ophidiomycosis is a native disease to Europe. To help answer this question, preserved specimens within the wet collections of natural history museums across Great Britain (and elsewhere in Europe) should be sampled. Natural history collections throughout Europe hold a large number of specimens, which cover a long period of time (dating back to the middle of the 19th century), potentially allowing the determination of when and where ophidiomycosis was first detected. This has already been completed for two collections in the United States with protocols for such work (Lorch *et al*., 2021), and more recently in Switzerland and Italy (Origgi *et al*., 2022). Where positive samples are found, these should be sequenced and compared to the strains analysed by Ladner *et al*. (2022). The detection of pathogen DNA is possible from museum specimens that have been stored in both formalin and ethanol (Soto-Azat *et al*., 2009; Origgi *et al*., 2022), as is the sequencing of such DNA (Ruane & Austin, 2017). This would assist in building a better understanding of the origins and relationships of *Oo* strains in Europe.

The current ongoing efforts to digitise the natural history collections in the United Kingdom and across Europe (Blagoderov *et al*., 2012; Addink *et al*., 2019), may allow researchers to better target *Oo* detection in wet specimens. With the associated metadata provided, sites where snakes of all species that have been confirmed to be affected by ophidiomycosis can be screened using these historic samples to see if, and when, emergence occurred throughout Europe. Photographs taken in the field of the affected barred grass snakes with skin lesions that were rt-PCR positive could be used a reference library, so that future researchers can better identify which specimens to sample, by comparing them to photos of specimens uploaded to digital databases. This could assist in identifying and quantifying those specimens with skin lesions, saving time and allowing for more accurate predictions of project budgets to be made.

6.2.2.2 Captive snakes

There is also a need to monitor captive snakes from around the world, and the trade in live snakes from those countries where *Oo* has been confirmed, to ensure that further strains do not spillover to wild populations. To date, only one mating type of *Oo* has been detected in Europe in wild snakes (Ladner *et al*., 2022), but more could potentially be introduced through poor public awareness, and a lack of biosecurity. More are likely present across *Oo*’s range as the fungus is consistent with a heterothallic mating system (Ni *et al*., 2011; Ladner *et al*., 2022). Two clades of *Oo* have been identified across Europe and North America (Franklinos *et al*., 2017), and wider surveillance is likely to uncover more.

6.2.3 Extended geographic coverage of wild snake surveillance

As the known distribution of *Oo* expands, the surveillance of wild snakes in Asia is needed to determine the relationships of the strains in the recently confirmed cases there (Sun *et al.*, 2021; Takami *et al*., 2021), and how these relate to those in Europe and North America. Asia is also home to the largest diversity of natricine snakes with 183/252 of the currently recognised species (Deepak *et al*., 2021), which inhabit similar environments to *N. helvetica*. These species and ecosystems should be prioritised as they are the most likely locations that *Oo* could be detected, if the environmental determinants observed in Europe and North America hold true across Asia. Surveillance should not be limited to Asia however. There are few natricine snakes inhabiting sub-Saharan Africa or Australia (Deepak *et al*., 2021), but if candidate species with a similar ecology can be identified there, then these should also be sampled for *Oo*. When detections elsewhere are made, these will assist in building an evidence base regarding the origins of *Oo*, and where the fungus is native to, especially if isolates can be collected and sequenced.

6.3 Survival modelling and detectability in the barred grass snake (*Natrix helvetica*)

Our modelling suggests that the presence of skin lesions reduces the apparent annual survival of *N. helvetica* by approximately half. To further explore these findings, individual snakes could be radio-tracked, as has previously been completed by other researchers (Ward *et al.*, 2017). This would allow for detailed information to be collected regarding the progression or resolution of skin lesions and habitat use (Horton & Letcher, 2008). One of the problems with such a study, is that radio tracking is extremely time intensive, which limits their use (Thomas *et al*., 2011).

The estimated detectability of snakes with skin lesions was also significantly higher than that of snakes without. This likely indicates changes in the behaviour of snakes affected by ophidiomycosis, increasing their likelihood of encountering a predator, or surveyor alike. This increase in detectability has been suggested in other studies (Lorch *et al*., 2015; Tetzlaff *et al*., 2017). This evidence suggests that the impacts on *N. helvetica* survival caused by ophidiomycosis may be primarily through indirect mortality, which is also supported by the larger percentage of snakes with concurrent skin lesions having trauma potentially indicative of failed predation attempts. Therefore, radio tracking could be used to quantify behavioural differences linked to the differences in survival rates which we have estimated.

6.4 Welfare and behavioural considerations for barred grass snake (*Natrix helvetica*) surveys

Through monitoring the response of *N. helvetica* to capture and processing, we have demonstrated that there is a significant difference between the repertoires of anti-predatory behaviours displayed at each phase of handling. Musking was by far the most frequently observed behaviour during capture, which through the use of an odorous and foul-tasting musk excretion is designed to make a would-be predator drop the snake immediately (Kissner *et al*., 2000). In the processing phase, musking was far less common, likely because it is often used upon initial contact with a predator. Conversely, death feigning was rarely seen the capture phase, but was observed in almost half of snakes in the processing phase. Death feigning is often the last resort for snakes, following further manipulation from a predator, and employed if musking was ineffective (Gregory *et al*., 2007). This is the first time that the differences between the occurrences of the defensive behaviours in *N. helvetica* were related to the presence of skin lesions.

We currently lack data on the metabolic costs of all of the observed behaviours on *N. helvetica*, and so these should be established by future researchers, along with biological means of testing for the levels of stress (such as glucocorticoid levels), in order to develop best practise guidance that minimises welfare impacts. These costs could be estimated using methods such as the dynamic body acceleration (Wilson *et al*., 2020), which can be used across vertebrate classes. These results will help to better inform interventions designed to minimise the impact of handling on wild snakes, especially considering that high levels of stress (that are linked to prolonged periods of handling and manipulation) may be linked to immunosuppression – making those snakes more susceptible to disease such as ophidiomycosis.

6.5 Barred grass snakes (*Natrix helvetica*) as bioindicators of wetland habitats

Being snakes that are typically found in wetlands, *N. helvetica* can also play another role in the bigger picture of environmental monitoring. As high trophic level predators, snakes are globally recognised as bioindicators of wetland contamination (Campbell *et al*., 2005; Haskins *et al*., 2019). Watermill Broad Nature Reserve was once a former gravel quarry, which has since been restored for the purposes of nature conservation, like many former open-cast mineral and commodity extraction sites within the United Kingdom. Due to their very nature, heavy metals from the soils within the quarries can leach into the water courses of the wetlands, causing potential environmental damage. These can then bioaccumulate in predators (such as snakes), causing adverse biological effects (Campbell & Campbell, 2001; Lemaire *et al*., 2018).

Traditionally, the liver is used as a target organ for heavy metal monitoring studies of vertebrate species (Frossard *et al*., 2019). Recent research utilising non-lethal tissue sampling has proven to be effective in Western tiger snakes (*Notechis scutatus occidentalis*), using scale clips to detect heavy metals (Lettoof *et al*., 2021). These authors used laser ablation with inductively coupled plasma-atomic emission spectroscopy and mass spectrometry (LA-ICP-MS), enabling them to quantify the concentrations of 19 metals and metalloids, that had chemically bound to the keratin within the skin of tiger snakes. Given that *N. helvetica* and *Notechis scutatus occidentalis* occupy very similar niches, it would be interesting to undertake such analyses at Watermill Broad and compare the results. The presence of elevated levels of heavy metals (such as lead and cadmium) in the tissues of reptiles has been linked to a decline in white blood cells (Yu *et al.*, 2011), which are vital for the immune response. Snakes with higher levels of bioaccumulated heavy metals, may be immunosuppressed and therefore more susceptible to disease.

There is evidence of further bioaccumulation within *Notechis scutatus occidentalis*, such as second generation anticoagulant rodenticides (SGARs), following the analysis of liver samples (Lettoof *et al*., 2020). Rodents are regularly eaten by *N. helvetica* (Brown *et al*., 2014), although the proportion of them in the diet of snakes will vary on the habitat, and availability of other prey species. Therefore, if post-mortem examinations are conducted on snake carcasses, samples from the liver and other relevant tissues could also be analysed for the detection of environmental pollutants, such as SGARs. This would allow for similar conclusions to be drawn as Lettoof *et al*. (2020), in that non-target species may be exposed to potentially lethal doses of SGARs through trophic cascades.

These analyses will also help to determine whether or not the presence of environmental contaminants linked to the land use around Watermill Broad Nature Reserve and other such sites (agriculture), or those released into the environment through their previous use (quarries), increases the likelihood of the presence of skin lesions consistent with ophidiomycosis in *N. helvetica*.

6.6 Communicating the risks of ophidiomycosis

There are a number of changes that could be made to help limit the impact and potential spread of ophidiomycosis on wild snake populations, until we know more about its distribution throughout Great Britain, and the wider global landscape. To assist with this, a series of information leaflets (such as that produced by Garden Wildlife Health: https://www.gardenwildlifehealth.org/wp-content/uploads/sites/12/2017/07/Reptile-Snake-Fungal-Disease-factsheet-GWH.pdf) and infographics are needed to help raise awareness, and to disseminate the risks to those that can have the most positive impact. This information should also be shared more widely among the general public, so that if dead snakes with skin lesions are found, that they are submitted to projects such as Garden Wildlife Health for post-mortem examination. Reptile hobbyists should also be targeted so that they are aware of ophidiomycosis, the clinical signs associated with disease, and what to do if they suspect one of their snakes is affected. There may also be more than one infectious disease affecting wild snakes within Great Britain, so this targeted communication may assist in the detection of these additional pathogens also.

6.7 Conclusion

The key findings of potential concern from the results presented within this thesis are the prevalence of skin lesions in the study population of *N. helvetica*, and the reduced level of survival associated with them. These results indicate that ophidiomycosis may pose a threat to barred grass snake populations, and potentially other European natricine species. By studying the impact of a potentially fatal disease in a comparatively neglected species, and raising the profile of ophidiomycosis in the region, declines in other natricine snakes throughout Europe (and Asia given its high diversity of species), could also be explored with a disease framework in mind. National and international surveys are required in order to determine the long-term effects of ophidiomycosis on snake populations globally.

References

Abdelfattah, A., Malacrino, A., Wisniewski, M., Cacciola, S. O. & Schena, L. (2018). Metabarcoding: A powerful tool to investigate microbial communities and shape future plant protection strategies. *Biological Control*, **120**, 1-10.

Addink, W., Koureas, D. & Rubio, A. C. (2019). DiSSCo as a new regional model for scientific collections in Europe. *Biodiversity Information Science and Standards*, **3(6)**, e37592.

Akaike, H. (1974). A new look at the statistical model identification. *IEEE Transactions on Automatic Control*, **19(6)**, 716-723.

Albert, C., Luque, G. M. & Courchamp, F. (2018). The twenty most charismatic species. *PLoS ONE*, **13(7)**, e0199149.

Allender, M. C., Dreslik, M., Wylie, S., Phillips, C., Wylie, D. B., Maddox, C., Delaney, M. A. & Kinsel, M. J. (2011). *Chrysosporium* sp. infection in eastern massasauga rattlesnakes. *Emerging Infectious Diseases*, **17(12)**, 2383.

Allender, M. C., Raudabaugh, D. B., Gleason, F. H. & Miller, A. N. (2015). The natural history, ecology, and epidemiology of *Ophidiomyces ophiodiicola* and its potential impact on free-ranging snake populations. *Fungal Ecology*, **17**, 187-196.

Allender, M. C., Hileman, E. T., Moore, J. & Tetzlaff, S. (2016). Detection of *Ophidiomyces*, the causative agent of snake fungal disease, in the Eastern Massasauga (*Sistrurus catenatus*) in Michigan, USA, 2014. *Journal of Wildlife Diseases* **52(3)**, 694-698.

Anderson, P. K., Cunningham, A. A., Patel, N. G., Morales, F. J., Epstein, P. R. & Daszak, P. (2004). Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. *Trends in Ecology & Evolution*, **19(10)**, 535-544.

Andreone, F., Cadle, J. E., Cox, N., Glaw, F., Nussbaum, R. A., Raxworthy, C. J., Stuart, S. N., Vallan, D. & Vences, M. (2005). Species Review of Amphibian Extinction Risks in Madagascar: Conclusions from the Global Amphibian Assessment. *Conservation Biology*, **19(6)**, 1790-1802.

Antonelli, A., Fry, C., Smith, R. J., Simmonds, M. S. J., Kersey, P. J., Pritchard, H. W., Abbo, M. S., Acedo, C., Adams, J., Ainsworth, A. M., Allkin, B., Annecke, W., Bachman, S. P., Bacon, K., Bárrios, S., Barstow, C., Battison, A., Bell, E., Bensusan, K., Bidartondo, M. I., Blackhall-Miles, R. J., Borrell, J. S., Brearley, F. Q., Breman, E., Brewer, R. F. A., Brodie, J., Cámara-Leret, R., Campostrini Forzza, R., Cannon, P., Carine, M., Carretero, J., Cavagnaro, T. R., Cazar, M.-E., Chapman, T., Cheek, M., Clubbe, C., Cockel, C., Collemare, J., Cooper, A., Copeland, A. I., Corcoran, M., Couch, C., Cowell, C., Crous, P., da Silva, M., Dalle, G., Das, D., David, J. C., Davies, L., Davies, N., De Canha, M. N., de Lirio, E. J., Demissew, S., Diazgranados, M., Dickie, J., Dines, T., Douglas, B., Dröge, G., Dulloo, M. E., Fang, R., Farlow, A., Farrar, K., Fay, M. F., Felix, J., Forest, F., Forrest, L. L., Fulcher, T., Gafforov, Y., Gardiner, L. M., Gâteblé, G., Gaya, E., Geslin, B., Gonçalves, S. C., Gore, C. J. N., Govaerts, R., Gowda, B., Grace, O. M., Grall, A., Haelewaters, D., Halley, J. M., Hamilton, M. A., Hazra, A., Heller, T., Hollingsworth, P. M., Holstein, N., Howes, M.-J. R., Hughes, M., Hunter, D., Hutchinson, N., Hyde, K., Iganci, J., Jones, M., Kelly, L. J., Kirk, P., Koch, H., Krisai-Greilhuber, I., Lall, N., Langat, M. K., Leaman, D. J., Leão, T. C., Lee, M. A., Leitch, I. J., Leon, C., Lettice, E., Lewis, G. P., Li, L., Lindon, H., Liu, J. S., Liu, U., Llewellyn, T., Looney, B., Lovett, J. C., Łuczaj, Ł., Lulekal, E., Maggassouba, S., Malécot, V., Martin, C., Masera, O. R., Mattana, E., Maxted, N., Mba, C., McGinn, K. J., Metheringham, C., Miles, S., Miller, J., Milliken, W., Moat, J., Moore, P. G. P., Morim, M. P., Mueller, G. M., Muminjanov, H., Negrão, R., Nic Lughadha, E., Nicolson, N., Niskanen, T., Nono Womdim, R., Noorani, A., Obreza, M., O'Donnell, K., O'Hanlon, R., Onana, J.-M., Ondo, I., Padulosi, S., Paton, A., Pearce, T., Pérez Escobar, O. A., Pieroni, A., Pironon, S., Prescott, T. A. K., Qi, Y. D., Qin, H., Quave, C. L., Rajaovelona, L., Razanajatovo, H., Reich, P. B., Rianawati, E., Rich, T. C. G., Richards, S. L., Rivers, M. C., Ross, A., Rumsey, F., Ryan, M., Ryan, P., Sagala, S., Sanchez, M. D., Sharrock, S., Shrestha, K. K., Sim, J., Sirakaya, A., Sjöman, H., Smidt, E.C., Smith, D., Smith, P., Smith, S. R., Sofo, A., Spence, N., Stanworth, A., Stara, K., Stevenson, P. C., Stroh, P., Suz, L. M., Tambam, B. B., Tatsis, E. C., Taylor, I., Thiers, B., Thormann, I., Trivedi, C., Twilley, D., Twyford, A. D., Ulian, T., Utteridge, T., Vaglica, V., Vásquez-Londoño, C., Victor, J., Viruel, J., Walker, B. E., Walker, K., Walsh, A., Way, M., Wilbraham, J., Wilkin, P., Wilkinson, T., Williams, C., Winterton, D., Wong, K. M., Woodfield-Pascoe, N., Woodman, J., Wyatt, L., Wynberg, R., Zhang, B. G. (2020). *State of the World's Plants and Fungi 2020*. Royal Botanic Gardens, Kew.

Appleby, L. G. (1971). *British snakes*. John Baker (Publishers) Ltd, London.

Araújo, M. B., Thuiller, W. & Pearson, R. G. (2006). Climate warming and the decline of amphibians and reptiles in Europe. *Journal of Biogeography*, **33(10)**, 1712-1728.

ARC Trust. (2021). *Introduction to the National Reptile Survey*. Amphibian and Reptile Conservation Trust, Bournemouth, England.

ARG UK. (2017). *ARG UK Advice Note 4: Amphibian Disease Precautions: A Guide for UK Fieldworkers*. Amphibian and Reptile Groups of the United Kingdom.

Augustine, L., Baskir, E., Kozlowski, C. P., Hammack, S., Elden, J., Wanner, M. D., Franklin, A. D. & Powell, D. M. (2022). Investigating welfare metrics for snakes at the Saint Louis Zoo. *Animals*, **12(3)**, 373.

Auliya, M., Altherr, S., Ariano-Sanchez, D., Baard, E. H., Brown, C., Brown, R. M., Cantu, J-C., Gentile, G., Gildenhuys, P., Henningheim, E., Hintzmann, J., Kanari, K., Krvavac, M., Lettink, M., Lippert, J., Luiselli, L., Nilson, G., Nguyen, T. Q., Nijman, V., Parham, J. F., Pasachnik, S. A., Pedrono, M., Rauhaus, A., Córdova, D. R., Sanchez, M-E., Schepp, U., van Schingen, M., Schneeweiss, N., Segniagbeto, G. H., Somaweera, R., Sy, E. Y., Türkozan, O., Vinke, S., Vinke, T., Vyas, R., Williamson, S. & Ziegler, T. (2016). Trade in live reptiles, its impact on wild populations, and the role of the European market. *Biological Conservation*, **204**, 103-119.

Baker, S. J., Haynes, E., Gramhofer, M., Stanford, K., Bailey, S., Christman, M., Frasca Jr, S., Ossiboff, R. J., Lobato, D. & Allender, M. C. (2019). Case definition and diagnostic testing for snake fungal disease. *Herpetological Review*, **50(2)**, 279-285.

References

Baker, J. M. R. & Allain, S. J. R. (2020). Photographic identification of individual barred grass snakes *Natrix helvetica*. *Transactions of the Norfolk & Norwich Naturalists' Society*, **53(1)**, 67-70.

Baker, S. J., Niemiller, M. L., Stites, A. J., Ash, K. T., Davis, M., Dreslik, M. J. & Phillips, C. A. (2020). Evaluation of environmental DNA to detect *Sistrurus catenatus* and *Ophidiomyces ophiodiicola* in crayfish burrows. *Conservation Genetics Resources*, **12(1)**, 13-15.

Balmford, A., Bennun, L., Ten Brink, B., Cooper, D., Côté, I. M., Crane, P., Dobson, A., Dudley, N., Dutton, I., Green, R. E., Gregory, R. D., Harrison, J., Kennedy, E. T., Kremen, C., Leader-Williams, N., Lovejoy, T. E., Mace, G., May, R., Mayaux, P., Morling, P., Phillips, J., Redford, K., Ricketts, T. H., Rodríguez, J. P., Sanjayan, Z. M., Schei, P. J., van Jaarsveld, A. S. & Walther, B. A. (2005). The convention on biological diversity's 2010 target. *Science*, **307(5707)**, 212-213.

Barber, D. M., Poole, V. A., Sanchez, C. R., Roady, P. & Allender, M. C. (2016). Snake fungal infection associated with *Fusarium* found in *Nerodia erythrogaster transversa* (Blotched Water Snake) in Texas, USA. *Herpetological Review*, **47(1)**, 39-42.

Barratt, D. G. (1997). Predation by house cats, *Felis catus* (L.), in Canberra, Australia. I. Prey composition and preference. *Wildlife Research*, **24(3)**, 263-277.

Bates, D., Maechler, M., Bolker, B. & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, **67(1)**, 1-48.

Beconi, H. C., Carosini, A. & Smith, P. (2019). Die Hard: How Paraguayan snakes avoid predation and live to tell the tale. *Herpetology Notes*, **12**, 513-518.

Beebee, T. J. C. & Griffiths, R. A. (2000). *Amphibians and reptiles: A natural history of the British herpetofauna*. HarperCollins, London.

Beebee, T. J. C. & Ratcliffe, S. (2018). Inferring status changes of three widespread British reptiles from NBN Atlas records. *Herpetological Bulletin*, **143**, 18-22.

Bell, T. P. (2009). A novel technique for monitoring highly cryptic lizard species in forests. *Herpetological Conservation and Biology*, **4(3)**, 415-425.

Bellard, C., Bertelsmeier, C., Leadley, P., Thuiller, W. & Courchamp, F. (2012). Impacts of climate change on the future of biodiversity. *Ecology Letters*, **15(4)**, 365-377.

Bendik, N. F., Morrison, T. A., Gluesenkamp, A. G., Sanders, M. S. *&* O'Donnell, L. J. (2013). Computer-assisted photo identification outperforms visible implant elastomers in an endangered salamander, *Eurycea tonkawae*. *PLoS ONE*, **8**, e59424.

Benstead, P. (2000). Practical wetland restoration: some recent experiences of the Royal Society for the protection of birds. *Landscape Research*, **25(3)**, 394-398.

Berger, L., Speare, R., Daszak, P., Green, D. E., Cunningham, A. A., Goggin, C. L., Slocombe, R., Ragan, M. A., Hyatt, A. D., McDonald, K. R., Hines, H. B., Lips, K. R., Marantelli, G. & Parkes, H. (1998). Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings of the National Academy of Sciences*, **95(15)**, 9031-9036.

Bertelsen, M. F., Crawshaw, G. J., Sigler, L. & Smith, D. A. (2005). Fatal cutaneous mycosis in tentacled snakes (*Erpeton tentaculatum*) caused by the *Chrysosporium* anamorph of *Nannizziopsis vriesii*. *Journal of Zoo and Wildlife Medicine*, **36(1)**, 82-87.

Bevan, E., Whiting, S., Tucker, T., Guinea, M., Raith, A. & Douglas, R. (2018). Measuring behavioral responses of sea turtles, saltwater crocodiles, and crested terns to drone disturbance to define ethical operating thresholds. *PLoS ONE*, **13(3)**, e0194460.

Bickford, D., Howard, S. D., Ng, D. J. & Sheridan, J. A. (2010). Impacts of climate change on the amphibians and reptiles of Southeast Asia. *Biodiversity and Conservation*, **19(4)**, 1043-1062

Bland, L. M. & Böhm, M. (2016). Overcoming data deficiency in reptiles. *Biological Conservation*, **204**, 16-22.

Blagoderov, V., Kitching, I. J., Livermore, L., Simonsen, T. J. & Smith, V. S. (2012). No specimen left behind: industrial scale digitization of natural history collections. *ZooKeys*, **209**, 133-146.

Blouin-Demers, G., Prior, K. A. & Weatherhead, P. J. (2002). Comparative demography of black rat snakes (*Elaphe obsoleta*) in Ontario and Maryland. *Journal of Zoology*, **256(1)**, 1-10.

Bobadilla Suarez, M., Ewen, J. G., Groombridge, J. J., Beckmann, K., Shotton, J., Masters, N., Hopkins, T. & Sainsbury, A. W. (2017). Using qualitative disease risk analysis for herpetofauna conservation translocations transgressing ecological and geographical barriers. *EcoHealth*, **14(1)**, 47-60.

Böhm, M., Collen, B., Baillie, J. E. M., Bowles, P., Chanson, J., Cox, N., Hammerson, G., Hoffmann, M., Livingstone, S. R., Ram, M., Rhodin A. G. J., Stuart, S. N., van Dijk, P. P., Young, B. E., Afuang, L. E., Aghasyan, A., García, A., Aguilar, C., Ajtic, R., Akarsu, F., Alencar, L. R. V., Allison, A., Ananjeva, N., Anderson, S., Andrén, C., Ariano-Sánchez, D., Arredondo, J. C., Auliya, M., Austin, C. C., Avci, A., Baker, P. J., Barreto-Lima, A. F., Barrio-Amorós, C. L., Basu, D., Bates, M. F., Batistella, A., Bauer, A., Bennett, D., Böhme, W., Broadley, D., Brown, R., Burgess, J., Captain, A., Carreira, S., Castañeda, M. D. R., Castro, F., Catenazzi, A., Cedeño-Vázquez, J. R., Chapple, D. G., Cheylan, M., Cisneros-Heredia, D. F., Cogalniceanu, D., Cogger, H., Corti, C., Costa, G. C., Couper, P. J., Courtney, T., Crnobrnja-Isailovic, J., Crochet, P-A., Crother, B., Cruz, F., Daltry, J. C., Daniels, R. J. R., Das, I., de Silva, A., Diesmos, A. C., Dirksen, L., Doan, T.M., Dodd, C. K., Doody, J. S., Dorcas, M. E., Duarte de Barros Filho, J., Egan, V. T., El Mouden, E. H., Embert, D., Espinoza, R. E., Fallabrino, A., Feng, X., Feng, Z-J., Fitzgerald, L., Flores-Villela, O., França, F. G. R., Frost, D., Gadsden, H., Gamble, T., Ganesh, S. R., Garcia, M. A., García-Pérez, J. E., Gatus, J., Gaulke, M., Geniez, P., Georges, A., Gerlach, J., Goldberg, S., Gonzalez, J-C. T., Gower, D. J., Grant, T., Greenbaum, E., Grieco, C., Guo, P., Hamilton, A. M., Hare, K., Hedges, S. B., Heideman, N., Hilton-Taylor, C., Hitchmough, R., Hollingsworth, B., Hutchinson, M., Ineich, I., Iverson, J., Jaksic, F. M., Jenkins, R., Joger, U., Jose, R., Kaska, Y., Kaya, U., Keogh, J. S., Köhler, G., Kuchling, G., Kumlutaş Y., Kwet, A., La Marca, E., Lamar, W., Lane, A., Lardner, B., Latta, C., Latta, G., Lau, M., Lavin, P., Lawson, D., LeBreton, M., Lehr, E., Limpus, D., Lipczynski, N., Lobo, A. S., López-Luna, M. A., Luiselli, L., Lukoschek, V., Lundberg, M., Lymberakis, P., Macey, R., Magnusson, W. E., Mahler, D. L., Malhotra, A., Mariaux, J., Maritz, B., Marques, O. A. V., Márquez, R., Martins, M., Masterson, G., Mateo, J. A., Mathew, R., Mathews, N., Mayer, G., McCranie, J. R., Measey, G. J., Mendoza-Quijano, F., Menegon, M., Métrailler, S., Milton, D. A., Montgomery, C., Morato, S. A. A., Mott, T., Muñoz-Alonso, A., Murphy, J., Nguyen, T. Q., Nilson, G., Nogueira, C., Núñez, H., Orlov, N., Ota, H., Ottenwalder, J., Papenfuss, T., Pasachnik, S., Passos, P., Pauwels, O. S. G., Pérez-Buitrago, N., Pérez-Mellado, V., Pianka, E. R., Pleguezuelos, J., Pollock, C., Ponce-Campos, P., Powell, R., Pupin, F., Quintero Díaz, G. E., Radder, R., Ramer, J., Rasmussen, A. R., Raxworthy, C., Reynolds, R., Richman, N., Rico, E. L., Riservato, E., Rivas, G., da Rocha, P. L. B., Rödel, M-O., Rodríguez Schettino, L., Roosenburg, W. M., Ross, J. P., Sadek, R., Sanders, K., Santos-Barrera, G., Schleich, H. H., Schmidt, B. R., Schmitz, A., Sharifi, M., Shea, G., Shi, H.-T., Shine, R., Sindaco, R., Slimani, T., Somaweera, R., Spawls, S., Stafford, P., Stuebing, R., Sweet, S., Sy, E., Temple, H. J., Tognelli, M. F., Tolley, K., Tolson, P. J., Tuniyev, B., Tuniyev, S., Üzüm, N., van Buurt, G., Van Sluys, M., Velasco, A., Vences, M., Veselý, M., Vinke, S., Vinke, T., Vogel, G., Vogrin, M., Vogt, R. C., Wearn, O. R., Werner, Y. L., Whiting, M. J., Wiewandt, T., Wilkinson, J., Wilson, B., Wren, S., Zamin, T., Zhou, K., Zug, G. (2013). The conservation status of the world's reptiles. *Biological Conservation*, **157**, 372-385.

Bohuski, E., Lorch, J. M., Griffin, K. M. & Blehert, D. S. (2015). TaqMan real-time polymerase chain reaction for detection of *Ophidiomyces ophiodiicola*, the fungus associated with snake fungal disease. *BMC Veterinary Research*, **11(1)**, 95.

Bolger, D. T., Morrison, T. A., Vance, B., Lee, D. & Farid, H. (2012). A computer-assisted system for photographic mark–recapture analysis. *Methods in Ecology and Evolution*, **3(5)**, 813-822.

Borczyk, B. (2004). Causes of mortality and bodily injury in grass snakes (*Natrix natrix*) from the „Stawy Milickie" nature reserve (SW Poland). *Herpetological Bulletin*, **90**, 22-26.

Boulenger, G. A. (1913). *The Snakes of Europe*. Methuen & Company Limited, London.

Bourret, V., Gutiérrez López, R., Melo, M. & Loiseau, C. (2021). Metabarcoding options to study eukaryotic endoparasites of birds. *Ecology and Evolution*, **11(16)**, 10821-10833.

Bowman, M. R., Paré, J. A., Sigler, L., Naeser, J. P., Sladky, K. K., Hanley, C. S., Helmer, P., Phillips, L. A., Brower, A. & Porter, R. (2007). Deep fungal dermatitis in three inland bearded dragons (*Pogona vitticeps*) caused by the *Chrysosporium* anamorph of *Nannizziopsis vriesii*. *Medical Mycology*, **45(4)**, 371-376.

Boyle, D. G., Boyle, D. B., Olsen, V., Morgan, J. A. T. & Hyatt, A. D. (2004). Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Diseases of Aquatic Organisms*, **60(2)**, 141-148.

Brattstrom, B. H. (1965). Body temperatures of reptiles. *American Midland Naturalist*, **73(2)**, 376-422.

Breininger, D. R., Mazerolle, M. J., Bolt, M. R., Legare, M. L., Drese, J. H. & Hines, J. E. (2012). Habitat fragmentation effects on annual survival of the federally protected eastern indigo snake. *Animal Conservation*, **15(4)**, 361-368.

Briones, V., Téllez, S., Goyache, J., Ballesteros, C., del Pilar Lanzarot, M., Domínguez, L. & Fernández-Garayzábal, J. F. (2004). *Salmonella* diversity associated with wild reptiles and amphibians in Spain. *Environmental Microbiology*, **6(8)**, 868-871.

Brischoux, F., Dupoué, A., Lourdais, O. & Angelier, F. (2016). Effects of mild wintering conditions on body mass and corticosterone levels in a temperate reptile, the aspic viper (*Vipera aspis*). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, **192**, 52-56.

Brook, B. W., Sodhi, N. S. & Ng, P. K. (2003). Catastrophic extinctions follow deforestation in Singapore. *Nature*, **424(6947)**, 420-423.

Brooks, T. M., Mittermeier, R. A., Mittermeier, C. G., da Fonseca, G. A. B., Rylands, A. B., Konstant, W. R., Flick, P., Pilgrim, J., Oldfield, S., Magin, G. & Hilton-Taylor, C. (2002). Habitat loss and extinction in the hotspots of biodiversity. *Conservation Biology*, **16(4)**, 909-923.

Brown, E. E. (1956). Frequency of skin shedding in the northern banded water snake. *Herpetologica*, **12(4)**, 313-320.

Brown, D. S., Ebenezer, K. L. & Symondson, W. O. (2014). Molecular analysis of the diets of snakes: changes in prey exploitation during development of the rare smooth snake *Coronella austriaca*. *Molecular Ecology*, **23(15)**, 3734-3743.

Burbrink, F. T., Lorch, J. M. & Lips, K. R. (2017). Host susceptibility to snake fungal disease is highly dispersed across phylogenetic and functional trait space. *Science Advances*, **3(12)**, e1701387.

Burger, J., Zappalorti, R. T. & Gochfeld, M. (1987). Developmental effects of incubation temperature on hatchling pine snakes *Pituophis melanoleucus*. *Comparative Biochemistry and Physiology Part A: Physiology*, **87(3)**, 727-732.

Burnham, K. P. & Anderson, D. R. (2002). *Model section and multimodel inferences: A practical-theoretic approach*. Springer-Verlag.

Burns, G., Ramos, A. & Muchlinski, A. (1996). Fever response in North American snakes. *Journal of Herpetology*, **30(2)**, 133-139.

Bury, R. B. & Corn, P. S. (1987). Evaluation of pitfall trapping in northwestern forests: trap arrays with drift fences. *The Journal of Wildlife Management*, **51(1)**, 112-119.

Bury, S., Mazgajski, T. D., Najbar, B., Zając, B. & Kurek, K. (2020). Melanism, body size, and sex ratio in snakes—new data on the grass snake (*Natrix natrix*) and synthesis. *Die Naturwissenschaften*, **107(3)**, 22.

Byrd, A. L. & Segre, J. A. (2016). Adapting Koch's postulates. *Science*, **351(6270)**, 224-226.

Cabañes, F. J., Sutton, D. A. & Guarro, J. (2014). *Chrysosporium*-related fungi and reptiles: a fatal attraction. *PLoS Pathogens*, **10(10)**, e1004367.

Campbell, K. R. & Campbell, T. S. (2001). The accumulation and effects of environmental contaminants on snakes: a review. *Environmental Monitoring and Assessment*, **70(3)**, 253-301.

Campbell, K. R., Campbell, T. S. & Burger, J. (2005). Heavy metal concentrations in northern water snakes (*Nerodia sipedon*) from East Fork Poplar Creek and the Little River, East Tennessee, USA. *Archives of Environmental Contamination and Toxicology*, **49(2)**, 239-248.

Campbell, L. J., Burger, J., Zappalorti, R. T., Bunnell, J. F., Winzeler, M. E., Taylor, D. R. & Lorch, J. M. (2021). Soil reservoir dynamics of *Ophidiomyces ophidiicola*, the causative agent of snake fungal disease. *Journal of Fungi*, **7(6)**, 461.

Carlström, D. & Edelstam, C. (1946). Methods of marking reptiles for identification after recapture. *Nature*, **158(4021)**, 748-749.

Case, T. J. & Bolger, D. T. (1991). The role of introduced species in shaping the distribution and abundance of island reptiles. *Evolutionary Ecology*, **5(3)**, 272-290.

Ceballos, G., Ehrlich, P. R., Barnosky, A. D., García, A., Pringle, R. M. & Palmer, T. M. (2015). Accelerated modern human–induced species losses: Entering the sixth mass extinction. *Science Advances*, **1(5)**, e1400253.

Ceballos, G., Ehrlich, P. R. & Dirzo, R. (2017). Biological annihilation via the ongoing sixth mass extinction signaled by vertebrate population losses and declines. *Proceedings of the National Academy of Sciences*, **114(30)**, E6089-E6096.

Ceia-Hasse, A., Sinervo, B., Vicente, L. & Pereira, H. M. (2014). Integrating ecophysiological models into species distribution projections of European reptile range shifts in response to climate change. *Ecography*, **37(7)**, 679-688.

Chandler, H. C., Allender, M. C., Stegenga, B. S., Haynes, E., Ospina, E. & Stevenson, D. J. (2019). Ophidiomycosis prevalence in Georgia's eastern indigo snake (*Drymarchon couperi*) populations. *PLoS One*, **14(6)**, e0218351.

Ciesiołkiewicz, J., Orłowski, G., & Elżanowski, A. (2006). High juvenile mortality of grass snakes *Natrix natrix* (L.) on a suburban road. *Polish Journal of Ecology*, **54(3)**, 465-472.

Clark, J. A. & May, R. M. (2002). Taxonomic bias in conservation research. *Science*, **297(5579)**, 191-192.

Clark, R. W., Marchand, M. N., Clifford, B. J., Stechert, R. & Stephens, S. (2011). Decline of an isolated timber rattlesnake (*Crotalus horridus*) population: interactions between climate change, disease, and loss of genetic diversity. *Biological Conservation*, **144(2)**, 886-891.

Conn, P. B. & Cooch, E. G. (2009). Multistate capture–recapture analysis under imperfect state observation: an application to disease models. *Journal of Applied Ecology*, **46(2)**, 486-492.

Consul, A., Eger, S. & Kwet, A. (2009). The grass snake, *Natrix natrix natrix* (Squamata: Colubridae), as a predator of the Great Ramshorn Snail, *Planorbarius c. corneus* (Gastropoda: Planorbidae). *Salamandra*, **45(1)**, 50-52.

Cooke, A. S. & Arnold, H. R. (1982). National changes in status of the commoner British amphibians and reptiles before 1974. *British Journal of Herpetology*, **6**, 206-207.

Corn, J. L., Mertins, J. W., Hanson, B. & Snow, S. (2011). First reports of ectoparasites collected from wild-caught exotic reptiles in Florida. *Journal of Medical Entomology*, **48(1)**, 94-100.

Cox, N. A. & Temple, H. J. (2009). European red list of reptiles (No. 333.957094 E89e). IUCN, Gland (Suiza).

Cox, N., Young, B. E., Bowles, P., Fernandez, M., Marin, J., Rapacciuolo, G., Böhm, M., Brooks, T. M., Blair Hedges, S., Hilton-Taylor, C., Hoffman, M., Jenkins, R. K. B., Tognelli, M. F., Alexander, G. J., Allison, A., Ananjeva, N. B., Auliya, M., Avila, L. J., Chapple, D. G., Cisneros-Heredia, D. F., Cogger, H. G., Colli, G. R., de Silva, A., Eisemberg, C. C., Els, J., Fong G, A., Grant, T. D., Hitchmough, R. A., Iskandar, D. T., Kidera, N., Martins, M., Meiri, S., Mitchell, N. J., Molur, S., Nogueira, C. C., Ortiz, J. C., Penner, J., Rhodin, A. G. J., Rivas, G. A., Rödel, M.-O., Roll, U., Sanders, K. L., Santos-Barrera. G., Shea, G. M., Spawls, S., Stuart, B. L., Tolley, K. A., Trape, J.-F., Vidal, M. A., Wagner, P., Wallace, B. P. & Xie, Y. (2022). A global reptile assessment highlights shared conservation needs of tetrapods. *Nature*, **605**, 285-290.

Crawford, J. A., Dreslik, M. J., Baker, S. J., Phillips, C. A. & Peterman, W. E. (2020). Factors affecting the detection of an imperiled and cryptic species. *Diversity*, **12(5)**, 177.

Darwin, C. R. (1837-1838). *Notebook B*: [Transmutation of species].

Daszak, P., Cunningham, A. A. & Hyatt, A. D. (2000). Emerging infectious diseases of wildlife-threats to biodiversity and human health. *Science*, **287(5452)**, 443-449.

Daszak, P., Cunningham, A. A. & Hyatt, A. D. (2001). Anthropogenic environmental change and the emergence of infectious diseases in wildlife. *Acta Tropica*, **78(2)**, 103-116.

Davy, C. M., Kidd, A. G. & Wilson, C. C. (2015). Development and validation of environmental DNA (eDNA) markers for detection of freshwater turtles. *PLoS ONE*, **10(7)**, e0130965.

Davy, C. M., Shirose, L., Campbell, D., Dillon, R., McKenzie, C., Nemeth, N., Braithwaite, T., Cai, H., Degazio, T., Dobbie, T., Egan, S., Fotherby, H., Litzgus, J. D., Manorome, P., Marks, S., Paterson, J. E., Sigler, L., Slavic, D., Slavik, E., Urquhart, J. & Jardine, C. (2021). Revisiting ophidiomycosis (snake fungal disease) after a decade of targeted research. *Frontiers in Veterinary Science*, **8**, 665805.

Deepak, V., Maddock, S. T., Williams, R., Nagy, Z. T., Conradie, W., Rocha, S., Harris, D. J., Perera, A., Gvoždík, V., Doherty-Bone, T. M., Kamei, R. G., Menegon, M., Labisko, J., Morel, C., Cooper, N., Day, J. J. & Gower, D. J. (2021). Molecular phylogenetics of sub-Saharan African natricine snakes, and the biogeographic origins of the Seychelles endemic *Lycognathophis seychellensis*. *Molecular Phylogenetics and Evolution*, **161**, 107152.

Den Hartog, J. & Reijns, R. (2015). *I3 S Straighten Manual, ver. 1.0*. Free Software Foundation: Boston, MA, USA.

Denman, S.E. & McSweeney, C.S. (2005). Quantitative (real-time) PCR. In: Makkar, H.P.S. & McSweeney, C.S. (Eds.) *Methods in gut microbial ecology for ruminants*. The Netherlands: Springer. 105-115.

References

Dezetter, M., Le Galliard, J. F., Guiller, G., Guillon, M., Leroux-Coyau, M., Meylan, S., Brischoux, F., Angelier, F. & Lourdais, O. (2021). Water deprivation compromises maternal physiology and reproductive success in a cold and wet adapted snake *Vipera berus*. *Conservation Physiology*, **9(1)**, coab071.

Dezetter, M., Le Galliard, J. F., Leroux-Coyau, M., Brischoux, F., Angelier, F. & Lourdais, O. (2022). Two stressors are worse than one: combined heatwave and drought affect hydration state and glucocorticoid levels in a temperate ectotherm. *Journal of Experimental Biology*, **225(7)**, jeb243777.

Díaz-Delgado, J., Marrow, J. C., Flanagan, J. P., Bauer, K. L., Zhang, M., Rodrigues-Hoffmann, A., Groch, K. R., Gomez, G. & Balamayooran, G. (2020). Outbreak of *Paranannizziopsis australasiensis* infection in captive African bush vipers (*Atheris squamigera*). *Journal of Comparative Pathology*, **181**, 97-102.

Dibadj, B., Haynes, E., Vivirito, K., Wright, A., Stanford, K. & Allender, M. C. (2021). Investigating agreement between snake sheds and skin swabs in detection of *Ophidiomyces ophidiicola*. *Journal of Herpetological Medicine and Surgery*, **31(2)**, 119-123.

Dillon, R. M., Paterson, J. E., Manorome, P., Ritchie, K., Shirose, L., Slavik, E. & Davy, C. M. (2022). Seasonal and interspecific variation in the prevalence of *Ophidiomyces ophidiicola* and ophidiomycosis in a community of free-ranging Snakes. *Journal of Wildlife Diseases*, **58(4)**, 00134.

Dirzo, R., Young, H. S., Galetti, M., Ceballos, G., Isaac, N. J. & Collen, B. (2014). Defaunation in the Anthropocene. *Science*, **345(6195)**, 401-406.

Doan, T. M. (2003). Which methods are most effective for surveying rain forest herpetofauna? *Journal of Herpetology*, **37(1)**, 72-81.

Dohms, J. E. & Metz, A. (1991). Stress—mechanisms of immunosuppression. *Veterinary Immunology and Immunopathology*, **30(1)**, 89-109.

Dolinski, A. C., Allender, M. C., Hsiao, V. & Maddox, C. W. (2014). Systemic *Ophidiomyces ophiodiicola* infection in a free-ranging plains garter snake (*Thamnophis radix*). *Journal of Herpetological Medicine and Surgery*, **24(1)**, 7-10.

Doody J. S., Brauman R. J., Young J. E. & Fiorillo R. A. (1996). Death feigning in the mud snake *Farancia abacura* in captivity. *Herpetological Review*, **27(2)**, 82-83.

Doody, J. S., Roe, J., Mayes, P. & Ishiyama, L. (2009). Telemetry tagging methods for some freshwater reptiles. *Marine and Freshwater Research*, **60(4)**, 293-298.

Dorcas, M. E., Willson, J. D., Reed, R. N., Snow, R. W., Rochford, M. R., Miller, M. A., Meshaka, W. E., Andreadis, P. T., Mazzottie, F. J., Romagosai, C. M. & Hart, K. M. (2012). Severe mammal declines coincide with proliferation of invasive Burmese pythons in Everglades National Park. *Proceedings of the National Academy of Sciences*, **109(7)**, 2418-2422.

Dowling, H. G. (1951). A proposed standard system of counting ventrals in snakes. *British Journal of Herpetology*, **1(5)**, 97-99.

References

Driscoll, D. A. (2004). Extinction and outbreaks accompany fragmentation of a reptile community. *Ecological Applications*, **14(1)**, 220-240.

Dundas, S. J., Ruthrof, K. X., Hardy, G. E. S. J. & Fleming, P. A. (2019). Pits or pictures: a comparative study of camera traps and pitfall trapping to survey small mammals and reptiles. *Wildlife Research*, **46(2)**, 104-113.

Du Feu, C. R., Clark, J. A., Schaub, M., Fiedler, W. & Baillie, S. R. (2016). The EURING Data Bank–a critical tool for continental-scale studies of marked birds. *Ringing & Migration* **31(1)**, 1-18.

Duffus, A. L. J, Hughes, D. F., Kautz, A., Allain, S. J. R, & Meshaka Jr, W. E. (2022). Repeated sampling of wild individuals reveals *Ophidiomyces ophidiicola* infection dynamics in a Pennsylvania snake assemblage. *Journal of Wildlife Diseases*, **58(2)**, 290-297.

Durkin, J. (2016). Reptile Atlas of North-East England. 2016. Durham Nature.

Durso, A. M., Willson, J. D., & Winne, C. T. (2011). Needles in haystacks: estimating detection probability and occupancy of rare and cryptic snakes. *Biological Conservation*, **144(5)**, 1508-1515.

Durso, A. M. & Seigel, R. A. (2015). A snake in the hand is worth 10,000 in the bush. *Journal of Herpetology*, **49(4)**, 503-506.

Džukić, G. & Kalezić, M. L. (2004). *The biodiversity of amphibians and reptiles in the Balkan Peninsula*. In Balkan Biodiversity (pp. 167-192). Springer, Dordrecht.

Edwards, W. J. & Edwards, C. T. (2011) Population Limiting Factors. *Nature Education Knowledge*, **3(10)**, 1-4.

Enge, K. M. (2001). The pitfalls of pitfall traps. *Journal of Herpetology*, **35(3)**, 467-478.

Engelstoft, C. & Ovaska, K. E. (2000). Artificial cover-objects as a method for sampling snakes (*Contia tenuis* and *Thamnophis* spp.) in British Columbia. *Northwestern Naturalist*, **81(1)**, 35-43.

Engeman, R., Jacobson, E., Avery, M. L. & Meshaka Jr, W. E. (2011). The aggressive invasion of exotic reptiles in Florida with a focus on prominent species: A review. *Current Zoology*, **57(5)**, 599-612.

Epstein, P. R. (2001). Climate change and emerging infectious diseases. *Microbes and Infection*, **3(9)**, 747-754.

Falaschi, M., Manenti, R., Thuiller, W. & Ficetola, G. F. (2019). Continental-scale determinants of population trends in European amphibians and reptiles. *Global Change Biology*, **25(10)**, 3504-3515.

Farrell, T. M., Walden, H. D., & Ossiboff, R. J. (2023). The invasive pentastome *Raillietiella orientalis* in a banded water snake from the pet trade. *Journal of Veterinary Diagnostic Investigation*, **35(2)**, 201-203.

Fisher, M. C. & Garner, T. W. J. (2007). The relationship between the emergence of *Batrachochytrium dendrobatidis*, the international trade in amphibians and introduced amphibian species. *Fungal Biology Reviews*, **21(1)**, 2-9.

Fisher, M. C. & Garner, T. W. (2020). Chytrid fungi and global amphibian declines. *Nature Reviews Microbiology*, **18(6)**, 332-343.

Fisher, M. C., Garner, T. W. J. & Walker, S. F. (2009a). Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. *Annual Review of Microbiology*, **63(1)**, 291-310.

Fisher, M. C., Bosch, J., Yin, Z., Stead, D. A., Walker, J., Selway, L., Brouwn, A. J. P., Walker, L. A., Gow, N. A. R., Stajich, J. E. & Garner, T. W. J. (2009b). Proteomic and phenotypic profiling of the amphibian pathogen *Batrachochytrium dendrobatidis* shows that the genotype is linked to virulence. *Molecular Ecology*, **18(3)**, 415–429.

Fisher, M. C., Henk, D. A., Briggs, C. J., Brownstein, J. S., Madoff, L. C., McCraw, S. L. & Gurr, S. J. (2012). Emerging fungal threats to animal, plant and ecosystem health. *Nature,* **484(7393)**, 186-194.

Fisher, M. C., Gow, N. A. & Gurr, S. J. (2016). Tackling emerging fungal threats to animal health, food security and ecosystem resilience. *Philosophical Transactions of the Royal Society B: Biological Sciences,* **371(1709)**, 20160332.

Fisher, M. C., Gurr, S. J., Cuomo, C. A., Blehert, D. S., Jin, H., Stukenbrock, E. H., Stajich, J. E., Kahmann, R., Boone, C., Denning, D. W., Gow, N. A. R., Klein, B. S., Kronstad, J. W., Sheppard, D. C., Taylor, J. W., Wright, G.D., Heitman, J., Casadevalle, A. & Cowen, L. E. (2020). Threats posed by the fungal kingdom to humans, wildlife, and agriculture. *MBio,* **11(3)**, e00449-20.

Fitzpatrick, L. D., Pasmans, F., Martel, A. & Cunningham, A. A. (2018). Epidemiological tracing of *Batrachochytrium salamandrivorans* identifies widespread infection and associated mortalities in private amphibian collections. *Scientific Reports,* **8**, 13845.

Foster, M. S. (2012). 'Standard Techniques for Inventory and Monitoring', in McDiarmid, R. W., Foster, M. S., Guyer, C., Chernoff, N., & Gibbons, J. W. (Ed.) *The Reptile Biodiversity*. University of California Press, 205-272.

Foster, J., Driver, D., Ward, R. & Wilkinson, J. (2021). IUCN Red List assessment of amphibians and reptiles at Great Britain and country scale. Report to Natural England. ARC report. ARC, Bournemouth.

Fox, J. & Monette, G. (1992). Generalized collinearity diagnostics. *Journal of the American Statistical Association*, **87(417)**, 178-183.

Francioli, D., Lentendu, G., Lewin, S. & Kolb, S. (2021). DNA metabarcoding for the characterization of terrestrial microbiota—pitfalls and solutions. *Microorganisms,* **9(2)**, 361.

Francq, E. N. (1969). Behavioral aspects of feigned death in the opossum *Didelphis marsupialis*. *American Midland Naturalist,* **81(2)**, 556-568.

Frankham, R., Briscoe, D. A. & Ballou, J. D. (2002). *Introduction to conservation genetics*. Cambridge University Press.

Franklinos, L. H. V., Lorch, J. M., Bohuski, E., Fernandez, J. R. R., Wright, O. N., Fitzpatrick, L., Petrovan, S., Durrant, C., Linton, C., Baláž, V., Cunningham, A. A. &

Lawson, B. (2017). Emerging fungal pathogen *Ophidiomyces ophiodiicola* in wild European snakes. *Scientific Reports*, **7**, 3844.

Freret-Meurer, N. V., Fernandez, T. C., Lopes, D. A., Vaccani, A. C. & Okada, N. B. (2017). Thanatosis in the Brazilian seahorse *Hippocampus reidi* Ginsburg, 1933 (Teleostei: Syngnathidae). *Acta Ethologica*, **20(1)**, 81-84.

Frossard, A., Leite, F. L., Silva, E. L., Carneiro, M. T., Júnior, J. L., Gomes, L. C. & Endringer, D. C. (2019). The snake *Bothrops jararaca* (Squamata: Viperidae) is a suitable bioindicator of environmental exposure to cadmium: an experimental study. *Ecological Indicators*, **104**, 166-171.

Fuentes, M. M. P. B., Limpus, C. J. & Hamann, M. (2011). Vulnerability of sea turtle nesting grounds to climate change. *Global Change Biology*, **17(1)**, 140-153.

Fuentes, R., Castillo, M., Belton, E., Zambrano, E., Quintero, H. & Batista, A. (2021). Dead snake! A strategy for survival: Thanatosis in some Panamanian snakes with a review of death-feigning in American snakes. *Reptiles & Amphibians*, **28(3)**, 389-396.

Gallup, G. G., Nash, R. F., Donegan, N. H. & McClure, M. K. (1971). The immobility response: A predator-induced reaction in chickens. *The Psychological Record*, **21(4)**, 513-519.

Gally, M., Silva, A. S. F. L. & Zina, J. (2012). Death feigning in *Physalaemus kroyeri* (Reinhardt and Lütken, 1862) (Anura, Leiuperidae). *Herpetology Notes*, **5**, 133-135.

Garcia, R. A., Cabeza, M., Rahbek, C. & Araújo, M. B. (2014). Multiple dimensions of climate change and their implications for biodiversity. *Science*, **344(6183)**, 1247579.

Gardner, E., Julian, A., Monk, C. & Baker, J. (2019). Make the Adder Count: population trends from a citizen science survey of UK adders. *Herpetological Journal*, **29(1)**, 57-70.

Gärdenfors, U., Hilton-Taylor, C., Mace, G. M. & Rodriguez, J. P. (2001). The application of IUCN Red List criteria at regional levels. *Conservation Biology*, **15(5)**, 1206-1212.

Gatto, C. R., Rotger, A., Robinson, N. J. & Tomillo, P. S. (2018). A novel method for photo-identification of sea turtles using scale patterns on the front flippers. *Journal of Experimental Marine Biology and* Ecology, **506**, 18-24.

Gent, T. & Gibson, S. (1998). *Herpetofauna Workers Manual*. Joint Nature Conservation Committee (JNCC), Peterborough.

Gent, A. H. & Spellerberg, I. F. (1993). Movement rates of the smooth snake *Coronella austriaca* (Colubridae): a radio-telemetric study. *Herpetological Journal*, **3(4)**, 140-146.

Germano, J. M., Field, K. J., Griffiths, R. A., Clulow, S., Foster, J., Harding, G. & Swaisgood, R. R. (2015). Mitigation-driven translocations: are we moving wildlife in the right direction? *Frontiers in Ecology and the Environment*, **13(2)**, 100-105.

Gibbons, J. W., Scott, D. E., Ryan, T. J., Buhlmann, K. A., Tuberville, T. D., Metts, B. S., Greene, J. L., Mills, T., Leiden, Y., Poppy, S. & Winne, C. T. (2000). The global decline of reptiles, Déjà Vu Amphibians: reptile species are declining on a global scale. Six significant threats to reptile populations are habitat loss and degradation, introduced invasive species, environmental pollution, disease, unsustainable use, and global climate change. *AIBS Bulletin*, **50(8),** 653-666.

Gimenez, O., Lebreton, J. D., Choquet, R. & Pradel, R. (2018). R2ucare: An r package to perform goodness-of-fit tests for capture–recapture models. *Methods in Ecology and Evolution*, **9(7)**, 1749–1754.

Golubović, A., Anđelković, M., Tomović, L., Arsovski, D., Gvozdenović, S., Šukalo, G., Ajtić, R. & Bonnet, X. (2021). Death-feigning propensity varies within dice snake populations but not with sex or colour morph. *Journal of Zoology*, **314(3)**, 203-210.

Gregory, P. T. (2008). Bluffing and waiting: Handling effects and post-release immobility in a death-feigning snake (*Natrix natrix*). *Ethology*, **114(8)**, 768-774.

Gregory, P. T. (2013). Once bitten, twice shy: Does previous experience influence behavioural decisions of snakes in encounters with predators? *Ethology*, **119(11)**, 919-925.

Gregory, P. T. (2016). Responses of natricine snakes to predatory threat: a mini-review and research prospectus. *Journal of Herpetology*, **50(2)**, 183-195.

Gregory, P. T. & Isaac, L. A. (2004). Food habits of the grass snake in southeastern England: is *Natrix natrix* a generalist predator? *Journal of Herpetology*, **38(1)**, 88-95.

Gregory, P. T. & Isaac, L. A. (2005). Close encounters of the worst kind: patterns of injury in a population of grass snakes (*Natrix natrix*). *Herpetological Journal*, **15(4)**, 213-219.

Gregory, P. T., Isaac, L. A. & Griffiths, R. A. (2007). Death feigning by grass snakes (*Natrix natrix*) in response to handling by human "predators". *Journal of Comparative Psychology*, **121(2)**, 123.

Grioni, A., To, K. W., Crow, P., Rose-Jeffreys, L., Ching, K. K., Chu, L. O., Hill, F. I., Chan, H. K. & Cheung, K. S. (2021). Detection of *Ophidiomyces ophidiicola* in a wild Burmese python (*Python bivittatus*) in Hong Kong SAR, China. *Journal of Herpetological Medicine and Surgery*, **31(4)**, 283-291.

Grisnik, M., Leys, J. E., Bryan, D., Hardman, R. H., Miller, D. L., Cobb, V. A., Ogle, C., Simpson, C., Campbell, J. R., Applegate, R. D., Allender, M. C., Nordberg, E. J., Hoekstra, A. A. & Walker, D. M. (2018). Host and geographic range of snake fungal disease in Tennessee, USA. *Herpetological Review*, **49(4)**, 682-690.

Grogan, L. F., Skerratt, L. F., Berger, L., Cashins, S. D., Trengove, R. D. & Gummer, J. P. (2018). Chytridiomycosis causes catastrophic organism-wide metabolic dysregulation including profound failure of cellular energy pathways. *Scientific Reports*, **8**, 8188.

Guillera-Arroita, G., Lahoz-Monfort, J. J., MacKenzie, D. I., Wintle, B. A. & McCarthy, M. A. (2014). Ignoring imperfect detection in biological surveys is dangerous: A response to ‘fitting and interpreting occupancy models'. *PLoS ONE*, **9(7)**, e99571.

Hagman, M., Elmberg, J., Kärvemo, S. & Löwenborg, K. (2012). Grass snakes (*Natrix natrix*) in Sweden decline together with their anthropogenic nesting-environments. *The Herpetological Journal*, **22(3)**, 199-202.

Halliday, T. R. & Verrell, P. A. (1988). Body size and age in amphibians and reptiles. *Journal of Herpetology*, **22(3)**, 253-265.

Hartley, M. & Sainsbury, A. (2017). Methods of disease risk analysis in wildlife translocations for conservation purposes. *EcoHealth*, **14(1)**, 16-29.

Haskins, D. L., Gogal, R. M. & Tuberville, T. D. (2019). Snakes as novel biomarkers of mercury contamination: a review. *Reviews of Environmental Contamination and Toxicology* **249**, 133-152.

Haynes, E., Pohly, A., Clifford, D. L., Patterson, L. C., Manning, S., Wack, R. F. & Allender, M. C. (2021). First report of ophidiomycosis in a free-ranging California kingsnake (*Lampropeltis californiae*) in California, USA. *Journal of Wildlife Diseases*, **57(1)**, 246-249.

Harper, G. J., Steininger, M. K., Tucker, C. J., Juhn, D. & Hawkins, F. (2007). Fifty years of deforestation and forest fragmentation in Madagascar. *Environmental Conservation*, **34(4)**, 325-333.

Hernandez, P. A., Graham, C. H., Master, L. L. & Albert, D. L. (2006). The effect of sample size and species characteristics on performance of different species distribution modeling methods. *Ecography*, **29(5)**, 773-785.

HM Government. (2022). https://www.legislation.gov.uk/ukpga/1981/69. Accessed 01/07/22.

Hoekstra, L. A., Schwartz, T. S., Sparkman, A. M., Miller, D. A. & Bronikowski, A. M. (2020). The untapped potential of reptile biodiversity for understanding how and why animals age. *Functional Ecology*, **34(1)**, 38-54.

Hoon-Hanks, L. L., Ossiboff, R. J., Bartolini, P., Fogelson, S. B., Perry, S. M., Stöhr, A. C., Cross, S. T., Wellehen, J. F. X., Jacobson, E. R., Dubovi, E. J. & Stenglein, M. D. (2019). Longitudinal and cross-sectional sampling of serpentovirus (nidovirus) infection in captive snakes reveals high prevalence, persistent infection, and increased mortality in pythons and divergent serpentovirus infection in boas and colubrids. *Frontiers in Veterinary Science*, **6**, 338.

Horton, G. E. & Letcher, B. H. (2008). Movement patterns and study area boundaries: influences on survival estimation in capture–mark–recapture studies. *Oikos*, **117(8)**, 1131-1142.

Howard, S. D. & Bickford, D. P. (2014). Amphibians over the edge: silent extinction risk of Data Deficient species. *Diversity and Distributions*, **20(7)**, 837-846.

Howe, J. C. (1991). Field observations of death feigning in the convict tang, *Acanthurus triostegus* (Linnaeus), with comments on the nocturnal color pattern in juvenile specimens. *Journal of Aquariculture and Aquatic Sciences*, **4**, 13-15.

Hoyt, J. R., Kilpatrick, A. M. & Langwig, K. E. (2021). Ecology and impacts of white-nose syndrome on bats. *Nature Reviews Microbiology*, **19(3)**, 196-210.

Hudson, M. A., Young, R. P., Lopez, J., Martin, L., Fenton, C., McCrea, R., Griffiths, R. A., Adams, S.-L., Gray, G., Garcia, G. & Cunningham, A. A. (2016). In-situ itraconazole treatment improves survival rate during an amphibian chytridiomycosis epidemic. *Biological Conservation*, **195**, 37-45.

Huey, R. B., Deutsch, C. A., Tewksbury, J. J., Vitt, L. J., Hertz, P. E., Pérez, H. J. Á. & Garland, T. (2009). Why tropical forest lizards are vulnerable to climate warming. *Proceedings of the Royal Society of London B: Biological Sciences*, **276(1664)**, 1939-1948.

Humphreys, R. K. & Ruxton, G. D. (2018). A review of thanatosis (death feigning) as an anti-predator behaviour. *Behavioral Ecology and Sociobiology*, **72(2)**, 1-16.

Hyatt, A. D., Boyle, D. G., Olsen, V., Boyle, D. B., Berger, L., Obendorf, D., Dalton, A., Kriger, K., Hero, M., Hines, H., Phillot, R., Campbell, R., Marantelli, G., Gleason, F. & Colling, A. (2007). Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms*, **73(3)**, 175-192.

Idrisova, L. A. & Khairutdinov, I. Z. (2018). The Effect of Incubation Temperature on the Morphological Features of Grass Snake *Natrix natrix* (Linnaeus, 1758)(Ophidia: Colubridae). *Russian Journal of Herpetology*, **25(4)**, 283-292.

Inchuai, R., Weerakun, S., Nguyen, H. N. & Sukon, P. (2021). Global prevalence of chlamydial infections in reptiles: A systematic review and meta-analysis. *Vector-Borne and Zoonotic Diseases,* **21(1)**, 32-39.

Isaac, N. J. & Pocock, M. J. (2015). Bias and information in biological records. *Biological Journal of the Linnean Society*, **115(3)**, 522-531.

Janssen, J. & Krishnasamy, K. (2018). Left hung out to dry: How inadequate international protection can fuel trade in endemic species–The case of the earless monitor. *Global Ecology and Conservation*, **16**, e00464.

Jensen, M. P., Allen, C. D., Eguchi, T., Bell, I. P., LaCasella, E. L., Hilton, W. A., Hof, C. A. M. & Dutton, P. H. (2018). Environmental warming and feminization of one of the largest sea turtle populations in the world. *Current Biology*, **28(1)**, 154-159.

Jofré, G. M., Warn, M. R. & Reading, C. J. (2016). The role of managed coniferous forest in the conservation of reptiles. *Forest Ecology and Management*, **362**, 69-78.

Jones, R., Cable, J. & Bruford, M. W. (2008). An evaluation of non-invasive sampling for genetic analysis in northern European reptiles. *Herpetological Journal*, **18(1)**, 32-39.

Joppa, L. N., Williams, C. K., Temple, S. A. & Casper, G. S. (2009). Environmental factors affecting sampling success of artificial cover objects. *Herpetological Conservation and Biology*, **5(1)**, 143-148.

Jordan, R. (1970). Death-feigning in a captive red-bellied snake, *Storeria occipitomaculata* (Storer). *Herpetologica*, **26(4)**, 466-468.

Karesh, W. B., Cook, R. A., Bennett, E. L. & Newcomb, J. (2005). Wildlife trade and global disease emergence. *Emerging Infectious Diseases*, **11(7)**, 1000-1002.

Katz, E. M., Tolley, K. A. & Altwegg, R. (2013). Survival and abundance of Cape dwarf chameleons, *Bradypodion pumilum*, inhabiting a transformed, semi-urban wetland. *Herpetological Journal*, **23(4)**, 179-186.

Kearney, M., Shine, R. & Porter, W. P. (2009). The potential for behavioral thermoregulation to buffer "cold-blooded" animals against climate warming. *Proceedings of the National Academy of Sciences of the United States of America,* **106(10)**, 3835–3840.

Kéry, M. (2002). Inferring the absence of a species: a case study of snakes. *The Journal of Wildlife Management*, **66(2)**, 330-338.

Kéry, M. & Schmid, H. (2006). Estimating species richness: calibrating a large avian monitoring programme. *Journal of Applied Ecology*, **43(1)**, 101-110.

Kéry, M. & Schmidt, B. (2008). Imperfect detection and its consequences for monitoring for conservation. *Community Ecology*, **9(2)**, 207-216.

Khedkar, T., Sharma, R., Tiknaik, A., Khedkar, G., Naikwade, B. S., Ron, T. B., & Haymer, D. (2016). DNA barcoding using skin exuviates can improve identification and biodiversity studies of snakes. *Mitochondrial DNA Part A*, **27(1)**, 499-507.

Kindler, C., Chèvre, M., Ursenbacher, S., Böhme, W., Hille, A., Jablonski, D., Vamberger, M. & Fritz, U. (2017). Hybridization patterns in two contact zones of grass snakes reveal a new Central European snake species. *Scientific Reports*, **7(1)**, 7378.

King, R. B. & Turmo, J. R. (1997). The effects of ecdysis on feeding frequency and behavior of the common garter snake (*Thamnophis sirtalis*). *Journal of Herpetology*, **31(2)**, 310-312.

Kissner, K. J., Secoy, D. M. & Forbes, M. R. (1998). Sexual dimorphism in size of cloacal glands of the garter snake, *Thamnophis radix haydeni*. *Journal of Herpetology*, **32(2)**, 268-270.

Kissner, K. J., Blouin-Demers, G., & Weatherhead, P. J. (2000). Sexual dimorphism in malodorousness of musk secretions of snakes. *Journal of Herpetology*, **34(3)**, 491-493.

Kraus, F. (2015). Impacts from invasive reptiles and amphibians. *Annual Review of Ecology, Evolution, and Systematics*, **46**, 75-97.

Krysko, K. L., Burgess, J. P., Rochford, M. R., Gillette, C. R., Cueva, D., Enge, K. M., Somma, L. A., Stabile, J. L., Smith, D. C., Wasilewski, J. A., Kieckhefer III, G. N., Granatosky, M. C. & Nielsen, S. V. (2011). Verified non-indigenous amphibians and reptiles in Florida from 1863 through 2010: Outlining the invasion process and identifying invasion pathways and stages. *Zootaxa*, **3028(1)**, 1-64.

Laake, J., Rakhimberdiev, B. A., Turek, D. & McClintock, B. (2019). RMark. R package version 2.2.7.

Ladner, J. T., Palmer, J. M., Ettinger, C. L., Stajich, J. E., Farrell, T. M., Glorioso, B. M., Lawson, B., Price, S. J., Stengle, A. G., Grear, D. A. & Lorch, J. M. (2022). The population genetics of the causative agent of snake fungal disease indicate recent introductions to the USA. *PLoS Biology*, **20(6)**, e3001676.

Laking, A. E., Ngo, H. N., Pasmans, F., Martel, A. & Nguyen, T. T. (2017). *Batrachochytrium salamandrivorans* is the predominant chytrid fungus in Vietnamese salamanders. *Scientific Reports*, **7**, 44443.

Lambert, M. R., Womack, M. C., Byrne, A. Q., Hernández-Gómez, O., Noss, C. F., Rothstein, A. P., Blackburn, D. C., Collins, J. P., Crump, M. L., Koo, M. S., Nanjappa, P., Rollins-Smith, L., Vredenburg, V. T. & Rosenblum, E. B. (2020). Comment on “Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity”. *Science*, **367(6484)**, eaay1838.

Lande, R. (1998). Anthropogenic, ecological and genetic factors in extinction and conservation. *Population Ecology*, **40(3)**, 259-269.

Langton, T. E. S. (1989). *Snakes and lizards*. London, Whittet Books.

Lebreton, J. D., Burnham, K. P., Clobert, J. & Anderson, D. R. (1992). Modeling survival and testing biological hypotheses using marked animals: a unified approach with case studies. *Ecological Monographs*, **62(1)**, 67-118.

Lemaire, J., Bustamante, P., Olivier, A., Lourdais, O., Michaud, B., Boissinot, A., Galán, P. & Brischoux, F. (2018). Determinants of mercury contamination in viperine snakes, *Natrix maura*, in Western Europe. *Science of the Total Environment*, **635**, 20-25.

Lemm, J. M. & Tobler, M. W. (2021). Factors Affecting the Presence and Abundance of Amphibians, Reptiles, and Small Mammals under Artificial Cover in Southern California. *Herpetologica*, **77(4)**, 307-319.

Lenzen, M., Moran, D., Kanemoto, K., Foran, B., Lobefaro, L. & Geschke, A. (2012). International trade drives biodiversity threats in developing nations. *Nature*, **486(7401)**, 109-112.

Lettoof, D. C., Lohr, M. T., Busetti, F., Bateman, P. W. & Davis, R. A. (2020). Toxic time bombs: Frequent detection of anticoagulant rodenticides in urban reptiles at multiple trophic levels. *Science of the Total Environment*, **724**, 138218.

Lettoof, D. C., Rankenburg, K., McDonald, B. J., Evans, N. J., Bateman, P. W., Aubret, F. & Gagnon, M. M. (2021). Snake scales record environmental metal (loid) contamination. *Environmental Pollution*, **274**, 116547.

Leung, W. T., Thomas-Walters, L., Garner, T. W., Balloux, F., Durrant, C. & Price, S. J. (2017). A quantitative-PCR based method to estimate ranavirus viral load following normalisation by reference to an ultraconserved vertebrate target. *Journal of Virological Methods*, **249**, 147-155.

Lewis, S. L. & Maslin, M. A. (2015). Defining the Anthropocene. *Nature*, **519(7542)**, 171-180.

Lind, A. J., Welsh Jr, H. H. & Tallmon, D. A. (2005). Garter snake population dynamics from a 16-year study: Considerations for ecological monitoring. *Ecological Applications*, **15(1)**, 294-303.

Lind, C. M., Agugliaro, J., Lorch, J. M. & Farrell, T. M. (2022). Ophidiomycosis is related to seasonal patterns of reproduction, ecdysis, and thermoregulatory behavior in a free-living snake species. *Journal of Zoology*. Advance online publication.

Lindell, L. E. (1997). Annual variation in growth rate and body condition of adders, Vipera berus: effects of food availability and weather. *Canadian Journal of Zoology*, **75(2)**, 261-270.

Lion, M. B., Garda, A. A., Santana, D. J. & Fonseca, C. R. (2016). The conservation value of small fragments for Atlantic Forest reptiles. *Biotropica*, **48(2)**, 265-275.

Lips, K. R., Reeve, J. D. & Witters, L. R. (2003). Ecological traits predicting amphibian population declines in Central America. *Conservation Biology*, **17(4)**, 1078-1088.

Lips, K. R., Diffendorfer, J., Mendelson III, J. R. & Sears, M. W. (2008). Riding the wave: reconciling the roles of disease and climate change in amphibian declines. *PLoS Biology*, **6(3)**, e72.

Lock, M. M. G. & Griffiths, R. A. (2022). Detectability of reptiles in standardised surveys: a test using grass snake *Natrix helvetica* models. *Herpetological Journal*, **32(4)**, 183-189.

Longcore, J. E., Pessier, A. P. & Nichols, D. K. (1999). *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. *Mycologia*, **91(2)**, 219-227.

Lopes, C. M., Baêta, D., Sasso, T., Vanzetti, A., Raquel Zamudio, K., Taberlet, P. & Haddad, C. F. B. (2021). Power and limitations of environmental DNA metabarcoding for surveying leaf litter eukaryotic communities. *Environmental DNA*, **3(3)**, 528-540.

Lorch J. M., Lankton J., Werner K., Falendysz E. A., McCurley K. & Blehert D. S. (2015). Experimental infection of snakes with *Ophidiomyces ophiodiicola* causes pathological changes that typify snake fungal disease. *mBio*, **6**, e01534-15.

Lorch, J. M., Knowles, S., Lankton, J. S., Michell, K., Edwards, J. L., Kapfer, J. M., Staffen, R. A., Wild, E., Schmidt, K. Z., Ballmann, A. E., Blodgett, D., Farrell, T. M., Glorioso, B. M., Last, L. A., Price, S. J., Schuler, K. L., Smith, C. E., Wellehan, J. F. X. & Blehert, D. S. (2016). Snake fungal disease: an emerging threat to wild snakes. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **371(1709)**, 20150457.

Lorch, J. M., Price, S. J., Lankton, J. S. & Drayer, A. N. (2021). Confirmed cases of ophidiomycosis in museum specimens from as early as 1945, United States. *Emerging Infectious Diseases*, **27(7)**, 1986-1989.

Lowe, V. P. W. (1969). Population dynamics of the red deer (*Cervus elaphus* L.) on Rhum. *Journal of Animal Ecology*, **38(2)**, 425-457.

Lowe, D. (2004) Distinctive image features from scale-invariant keypoints. *International Journal of Computer Vision*, **60(2)**, 91–110.

Luiselli, L., Vignoli, L., Rugiero, L. & Meek, R. (2018). Declining occupancy rates in the hibernacula of aspic vipers (*Vipera aspis*) in Italy and France; evidence for climatic effects? *Herpetological Journal*, **28(4)**, 137-142.

Lutterschmidt, W. I. (1994). The effect of surgically implanted transmitters upon the locomotory performance of the checkered garter snake, *Thamnophis m. marcianus*. *Herpetological Journal*, **4(1)**, 11-14.

Lydeard, C., Cowie, R. H., Ponder, W. F., Bogan, A. E., Bouchet, P., Clark, S. A., Cummings, K. S., Frest, T. J., Gargominy, O., Herbert, D. G., Hershler, R., Perez, K. E., Roth, B., Seddon, M., Strong, E. E. & Thompson, F. G. (2004). The global decline of nonmarine mollusks. *BioScience*, **54(4)**, 321-330.

Maderson, P. F. A. (1965). Histological changes in the epidermis of snakes during the sloughing cycle. *Proceedings of the Zoological Society of London*, **146(1)**, 98-113.

Madsen, T. (1983). Growth rates, maturation and sexual size dimorphism in a population of grass snakes, *Natrix natrix*, in southern Sweden. *Oikos*, **40(2)**, 277-282.

Madsen, T. (1984). Movements, home range size and habitat use of radio-tracked grass snakes (*Natrix natrix*) in southern Sweden. *Copeia*, **1984(3)**, 707-713.

Madsen, T. (1987). Cost of reproduction and female life-history tactics in a population of grass snakes, *Natrix natrix*, in southern Sweden. *Oikos*, **49(2)**, 129-132.

Maigret, T. A. (2019). Snake scale clips as a source of high quality DNA suitable for RAD sequencing. *Conservation Genetics Resources*, **11(4)**, 373-375.

Major, T., Alkins, D. R., Jeffrey, L. & Wüster, W. (2020). Marking the un-markable: visible implant elastomer in wild juvenile snakes. *Herpetological Journal*, **30(3)**, 173-176.

Mandimbihasina, A. R., Woolaver, L. G., Concannon, L. E., Milner-Gulland, E. J., Lewis, R. E., Terry, A. M., Filzaha, T. N., Rabetafika, L. L. & Young, R. P. (2018). The illegal pet trade is driving Madagascar's ploughshare tortoise to extinction. *Oryx*, **54(2)**, 188-196.

Martel, A., Spitzen-van der Sluijs, A., Blooi, M., Bert, W., Ducatelle, R., Fisher, M. C., Woeltjes, A., Bosman, W., Chiers, K., Bossuyt, F. & Pasmans, F. (2013). *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. *Proceedings of the National Academy of Sciences*, **110(38)**, 15325-15329.

Martel, A., Blooi, M., Adriaensen, C., Van Rooij, P., Beukema, W., Fisher, M.C., Farrer, R. A., Schmidt, B. R., Tobler, U., Goka, K., Lips, K. R., Muletz, C., Zamudio, K. R., Bosch, J. Lötters, S., Wombwell, E., Garner, T. W. J., Cunningham, A. A., Spitzen-van der Sluijs, A., Salvidio, S., Ducatelle, R., Nishikawa, K., Nguyen, T. T., Kolby, J. E., Van Bocxlaer, I., Bossuyt, F. & Pasmans, F. (2014). Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. *Science*, **346(6209)**, 630-631.

Maritz, B., Penner, J., Martins, M., Crnobrnja-Isailović, J., Spear, S., Alencar, L. R., Sigala-Rodriguez, J., Messenger, K., Clark, R. W. & Soorae, P. (2016). Identifying global priorities for the conservation of vipers. *Biological Conservation*, **204**, 94-102.

Matthé, M., Sannolo, M., Winiarski, K., Spitzen-van der Sluijs, A., Goedbloed, D., Steinfartz, S. & Stachow, U. (2017). Comparison of photo-matching algorithms commonly used for photographic capture–recapture studies. *Ecology and Evolution*, **7(15)**, 5861-5872.

Mazerolle, M. J., Bailey, L. L., Kendall, W. L., Royle, J. A., Converse, S. J., & Nichols, J. D. (2007). Making great leaps forward: accounting for detectability in herpetological field studies. *Journal of Herpetology*, **41(4)**, 672-689.

McBride, M. P., Wojick, K. B., Georoff, T. A., Kimbro, J., Garner, M. M., Wang, X., Childress, A. L. & Wellehan, J. F. (2015). *Ophidiomyces ophiodiicola* dermatitis in eight free-ranging timber rattlesnakes (*Crotalus horridus*) from Massachusetts. *Journal of Zoo and Wildlife Medicine*, **46(1)**, 86-94.

McCallum, M. L. (1999). *Acris crepitans* (northern cricket frog). Death feigning. *Herpetological Review*, **30(2)**, 90.

McClenachan, L., Cooper, A. B., Carpenter, K. E. & Dulvy, N. K. (2012). Extinction risk and bottlenecks in the conservation of charismatic marine species. *Conservation Letters*, **5(1)**, 73-80.

McCoy, C. M., Lind, C. M. & Farrell, T. M. (2017). Environmental and physiological correlates of the severity of clinical signs of snake fungal disease in a population of pigmy rattlesnakes, *Sistrurus miliarius*. *Conservation Physiology*, **5**, cow077.

McDiarmid, R. W., Foster, M. S., Guyer, C., Chernoff, N. & Gibbons, J. W. (2012). *Reptile biodiversity: Standard methods for inventory and monitoring*. Oakland (CA), University of California Press.

McNeely, J. A. (1992). The sinking ark: pollution and the worldwide loss of biodiversity. *Biodiversity & Conservation*, **1(1)**, 2-18.

McKenzie, J. M., Price, S. J., Fleckenstein, J. L., Drayer, A. N., Connette, G. M., Bohuski, E., & Lorch, J. M. (2018). Field diagnostics and seasonality of *Ophidiomyces ophiodiicola* in wild snake populations. *EcoHealth*, **16(1)**, 141-150.

McKenzie, J. M., Price, S. J., Connette, G. M., Bonner, S. J. & Lorch, J. M. (2020). Effects of snake fungal disease on short-term survival, behavior, and movement in free-ranging snakes. *Ecological Applications*, **31(2)**, e2251.

Meier, G., Notomista, T., Marini, D. & Ferri, V. (2018). First case of Snake Fungal Disease affecting a free-ranging *Natrix natrix* (Linnaeus, 1758) in Ticino Canton, Switzerland. *Herpetology Notes*, **11**, 885-891.

Mertens, D. (1995). Population structure and abundance of grass snakes, *Natrix natrix*, in central Germany. *Journal of Herpetology*, **29(3)**, 454-456.

Miller, M. A., Kinsella, J. M., Snow, R. W., Hayes, M. M., Falk, B. G., Reed, R. N., Mazzotti, F. J., Guyer, C. & Romagosa, C. M. (2018). Parasite spillover: indirect effects of invasive Burmese pythons. *Ecology and Evolution*, **8(2)**, 830-840.Mingo, V., Lötters, S. & Wagner, N. (2016). Risk of pesticide exposure for reptile species in the European Union. *Environmental Pollution*, **215**, 164-169.

Monastersky, R. (2013). Global carbon dioxide levels near worrisome milestone. *Nature*, **497(7447)**, 13-14.

Moore, J. E., Scheiman, D. M. & Swihart, R. K. (2004). Field comparison of removal and modified double-observer modeling for estimating detectability and abundance of birds. *The Auk*, **121(3)**, 865-876.

Moreno-Rueda, G., Pleguezuelos, J. M., Pizarro, M. & Montori, A. (2012). Northward shifts of the distributions of Spanish reptiles in association with climate change. *Conservation Biology*, **26(2)**, 278-283.

Mori, A. & Burghardt, G. M. (2008). Comparative experimental tests of natricine antipredator displays, with special reference to the apparently unique displays in the Asian genus, *Rhabdophis*. *Journal of Ethology*, **26(1)**, 61-68.

Moro, D. & MacAulay, I. (2014). Computer-aided pattern recognition of large reptiles as a noninvasive application to identify individuals. *Journal of Applied Animal Welfare Science*, **17(2)**, 125-135.

Musah, Y., Attuquayefio, D. K., Pobee, A. N., & Holbech, L. H. (2021). Ophidiophobia, myth generation, and human perceptions: Implications for snake conservation in a typical savanna community of northern Ghana. *Human Dimensions of Wildlife,* **27(4)**, 321-344.

Mutoh, A. (1983). Death-feigning behavior of the Japanese colubrid snake *Rhabdophis tigrinus*. *Herpetologica*, **39(1)**, 78-80.

Nagy, Z. T., Sonet, G., Glaw, F. & Vences, M. (2012). First large-scale DNA barcoding assessment of reptiles in the biodiversity hotspot of Madagascar, based on newly designed COI primers. *PLoS ONE,* **7(3)**, e34506.

Nash, D. J., Humphries, N. & Griffiths, R. A. (2020). Effectiveness of translocation in mitigating reptile-development conflict in the UK. *Conservation Evidence,* **17**, 7-11.

Ni, M., Feretzaki, M., Sun, S., Wang, X. & Heitman, J. (2011). Sex in fungi. *Annual Review of Genetics,* **45**, 405-430.

Nichols, J. D. & Williams, B. K. (2006). Monitoring for conservation. *Trends in Ecology & Evolution*, **21(12)**, 668-673.

Norbury, G. (2001). Conserving dryland lizards by reducing predator-mediated apparent competition and direct competition with introduced rabbits. *Journal of Applied Ecology*, **38(6)**, 1350-1361.

Nordberg, E. J. & Schwarzkopf, L. (2015). Arboreal cover boards: using artificial bark to sample cryptic arboreal lizards. *Herpetologica,* **71(4)**, 268-273.

O'Hanlon, S. J., Rieux, A, Farrer, R. A., Rosa, G. M., Waldman, B., Bataille, A., Kosch, T. A., Murray, K. A., Brankovics, B., Fumagalli, M., Martin, M. D., Wales, N., Alvarado-Rybak, M., Bates, K. A., Berger, L., Böll, S., Brookes, L., Clare, F., Courtois, E. A., Cunningham. A. A., Doherty-Bone, T. M., Ghosh, P., Gower, D. J., Hintz, W. E., Höglund, J., Jenkinson, T. S., Lin, C-F., Laurila, A., Loyau, A., Martel, A., Meuling, A., Miaud, C., Minting, P., Pasmans, F., Schmeller, D. S., Schmidt, B. R., Shelton, J. M. G., Skerratt., L. F., Smith, F., Soto-Azat, C., Spagnoletti, M., Tessa, G., Toledo, L. F., Valenzuela-Sánchez, A., Verster, R., Vörös, J., Webb, R. J., Wierzbicki, C., Wombwell, E., Zamudio, K. R., Aanensen, D. M., James, T. Y., Gilbert, M. P. T., Weldon, C., Bosch, J., Balloux, F., Garner, T. W. J. & Fisher, M. C. (2018). Recent Asian origin of chytrid fungi causing global amphibian declines. *Science*, **360(6389),** 621-627.

Oliver, P. M., Adams, M., Lee, M. S. Y., Hutchinson, M. N. & Doughty, P. (2009). Cryptic diversity in vertebrates: molecular data double estimates of species diversity in a radiation of Australian lizards (*Diplodactylus*, Gekkota). *Proceedings of the Royal Society B: Biological Sciences*, **276**, 2001–2007.

Olson, D. H., Aanensen, D. M., Ronnenberg, K. L., Powell, C. I., Walker, S. F., Bielby, J., Garner, T. W. J., Weaver, G., The Bd Mapping Group & Fisher, M. C. (2013). Mapping the global emergence of *Batrachochytrium dendrobatidis*, the amphibian chytrid fungus. *PLoS ONE,* **8(2)**, e56802.

References

O'Mara, M. T., Wikelski, M. & Dechmann, D. K. (2014). 50 years of bat tracking: device attachment and future directions. *Methods in Ecology and Evolution*, **5(4)**, 311-319.

Origgi, F. C., Pisano, S. R., Glaizot, O., Hertwig, S. T., Schmitz, A. & Ursenbacher, S. (2022). *Ophiodimyces ophiodiicola*, Etiologic Agent of Snake Fungal Disease, in Europe since Late 1950s. *Emerging Infectious Diseases*, **28(10)**, 2064-2068.

Ovchinnikov, R. S., Vasilyev, D. B., Gaynullina, A. G., Yuzhakov, A. G., Kapustin, A. V., Savinov, V. A. & Gulyukin, A. M. (2021). Detection of *Ophidiomyces ophidiicola* in three file snakes (*Acrochordus granulatus*) imported from Indonesia to the Moscow Zoo (Russia). *Journal of Zoo and Wildlife Medicine*, **52(3)**, 1074-1078.

Paré, J. A. & Sigler, L. (2016). An overview of reptile fungal pathogens in the genera *Nannizziopsis*, *Paranannizziopsis*, and *Ophidiomyces*. *Journal of Herpetological Medicine and Surgery*, 26**(1-2)**, 46-53.

Patel, H., Naik, V. & Tank, S. K. (2016). Death-feigning behavior in two species of *Lygosoma* (Squamata: Scincidae) from India. *Phyllomedusa: Journal of Herpetology*, **15(2)**, 191-194.

Payne, J. L., Bush, A. M., Heim, N. A., Knope, M. L. & McCauley, D. J. (2016). Ecological selectivity of the emerging mass extinction in the oceans. *Science*, **353(6305)**, 1284-1286.

Peig, J. & Green, A. J. (2009). New perspectives for estimating body condition from mass/length data: the scaled mass index as an alternative method. *Oikos*, **118(12)**, 1883-1891.

Pimm, S. L., Russell, G. J., Gittleman, J. L., & Brooks, T. M. (1995). The future of biodiversity. *Science*, **269(5222)**, 347-350.

Pincheira-Donoso, D., Bauer, A. M., Meiri, S. & Uetz, P. (2013). Global taxonomic diversity of living reptiles. *PLoS ONE*, **8(3)**, e59741.

Piotrowski, J. S., Annis, S. L. & Longcore, J. E. (2004). Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. *Mycologia*, **96(1)**, 9-15.

Plummer, M. V. (1985). Demography of green snakes (*Opheodrys aestivus*). *Herpetologica*, **41(4)**, 373-381.

Pocock, M. J., Roy, H. E., Preston, C. D., & Roy, D. B. (2015). The Biological Records Centre: a pioneer of citizen science. *Biological Journal of the Linnean Society*, **115(3)**, 475-493.

Pounds, J. A., Bustamante, M. R., Coloma, L. A., Consuegra, J. A., Fogden, M. P., Foster, P. N., Marca, E. L., Masters, K. L., Merino-Viteri, A., Puschendorf, R., Ron, S. R., Sánchez-Azofeifa, S., Still, C. J. & Young, B. E. (2006). Widespread amphibian extinctions from epidemic disease driven by global warming. *Nature*, **439(7073)**, 161-167.

Price, S. J., Ariel, E., Maclaine, A., Rosa, G. M., Gray, M. J., Brunner, J. L. & Garner, T. W. (2017). From fish to frogs and beyond: Impact and host range of emergent ranaviruses. *Virology*, **511**, 272-279.

Price, S. J., Leung, W. T., Owen, C. J., Puschendorf, R., Sergeant, C., Cunningham, A. A., Balloux, F., Garner, T. W. J. & Nichols, R. A. (2019). Effects of historic and projected climate change on the range and impacts of an emerging wildlife disease. *Global Change Biology*, **25(8)**, 2648-2660.

QGIS.org. (2022). QGIS 3.12 Geographic Information System. QGIS Association.

R Core Team (2020). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Rainwater, K. L., Wiederhold, N. P., Sutton, D. A., Garner, M. M., Maguire, C., Sanders, C., Gibas, C., Cano, J. F., Guarro, J. & Stchigel, A. M. (2019). Novel *Paranannizziopsis* species in a Wagler's viper (*Tropidolaemus wagleri*), tentacled snakes (*Erpeton tentaculatum*), and a rhinoceros snake (*Rhynchophis boulengeri*) in a zoological collection. *Medical Mycology*, **57(7)**, 825-832.

Rajeev, S., Sutton, D. A., Wickes, B. L., Miller, D. L., Giri, D., Van Meter, M., Thompson, E. H., Rinaldi, M. G., Romanelli, A. M., Cano, J. F. & Guarro, J. (2009). Isolation and characterization of a new fungal species, *Chrysosporium ophiodiicola*, from a mycotic granuloma of a black rat snake (*Elaphe obsoleta obsoleta*). *Journal of Clinical Microbiology*, **47(4)**, 1264-1268.

Raske, M., Lewbart, G. A., Dombrowski, D. S., Hale, P., Correa, M. & Christian, L. S. (2012). Body temperatures of selected amphibian and reptile species. *Journal of Zoo and Wildlife Medicine*, **43(3)**, 517-521.

Raxworthy, C. J. & Nussbaum, R. A. (1994). A rainforest survey of amphibians, reptiles and small mammals at Montagne d'Ambre, Madagascar. *Biological Conservation*, **69**, 65-73.

Raxworthy C. J., Pearson R. G., Rabibisoa N., Rakotondrazafy A. M., Ramanamanjato J-B., Raselimanana A. P., Wu S., Nussbaum R. A. & Stone D. A. (2008). Extinction vulnerability of tropical montane endemism from warming and upslope displacement: a preliminary appraisal for the highest massif in Madagascar. *Global Change Biology*, **14(8)**, 1703–1720.

Reading, C. J. (1997). A proposed standard method for surveying reptiles on dry lowland heath. *Journal of Applied Ecology*, **34(4)**, 1057-1069.

Reading, C. J. & Davies, J. L. (1996). Predation by grass snakes (*Natrix natrix*) at a site in southern England. *Journal of Zoology*, **239(1)**, 73-82.

Reading, C. & Jofré, G. (2020). Smooth snake population decline and its link with prey availability. *Amphibia-Reptilia*, **41(1)**, 43-48.

Reading, C. J., Luiselli, L. M., Akani, G. C., Bonnet, X., Amori, G., Ballouard, J. M., Filipi, E., Naulleau, G. &, Pearson, D. & Rugiero, L. (2010). Are snake populations in widespread decline? *Biology Letters*, **6(6)**, 777-780.

Reardon, J. T., Whitmore, N., Holmes, K. M., Judd, L. M., Hutcheon, A. D., Norbury, G. & Mackenzie, D. I. (2012). Predator control allows critically endangered lizards to recover on mainland New Zealand. *New Zealand Journal of Ecology*, **36(2)**, 141-150.

Reed, R. N. (2005). An ecological risk assessment of nonnative boas and pythons as potentially invasive species in the United States. *Risk Analysis: An International Journal*, **25(3)**, 753-766.

Ribeiro-Júnior, M. A., Gardner, T. A. & Ávila-Pires, T. C. (2006). The effectiveness of glue traps to sample lizards in a tropical rainforest. *South American Journal of Herpetology*, **1(2)**, 131-137.

Ricciardi, A. & Cohen, J. (2007). The invasiveness of an introduced species does not predict its impact. *Biological Invasions*, **9(3)**, 309-315.

Riedle, J. D. (2014). Demography of an urban population of ring-necked snakes (*Diadophis punctatus*) in Missouri. *Herpetological Conservation and Biology*, **9(2)**, 278-284.

Riley, J. L., Baxter-Gilbert, J. H. & Litzgus, J. D. (2017). A comparison of three external transmitter attachment methods for snakes. *Wildlife Society Bulletin*, **41(1)**, 132-139.

Robinson, J. E., Griffiths, R. A., John, F. A. S. & Roberts, D. L. (2015). Dynamics of the global trade in live reptiles: Shifting trends in production and consequences for sustainability. *Biological Conservation*, **184**, 42-50.

Rodgers, C. J., Mohan, C. V. & Peeler, E. J. (2011). The spread of pathogens through trade in aquatic animals and their products. *Revue scientifique et technique (International Office of Epizootics)*, **30(1)**, 241-256.

Rodrigues, A. S., Pilgrim, J. D., Lamoreux, J. F., Hoffmann, M. & Brooks, T. M. (2006). The value of the IUCN Red List for conservation. *Trends in Ecology & Evolution*, **21(2)**, 71-76.

Rodríguez-Cabrera, T. M., Torres, J. & Romero, R. M. (2014). Body-inversion in the Cuban Racer, *Cubophis cantherigerus cantherigerus* (Dipsadidae): Death-feigning or warning signal? *Reptiles & Amphibians*, **21(3)**, 93-95.

Rogers, S. M. & Simpson, S. J. (2014). Thanatosis. *Current Biology*, **24(21)**, R1031-R1033.

Rosa, G., Guillaud, F., Priol, P. & Renet, J. (2020). Parameter affecting the I3S algorithm reliability: how does correcting for body curvature affect individual recognition? *Wildlife Research*, **48(1)**, 38-43.

Ruane, S. & Austin, C. C. (2017). Phylogenomics using formalin-fixed and 100+ year-old intractable natural history specimens. *Molecular Ecology Resources*, **17(5)**, 1003-1008.

Rugiero, L., Milana, G., Petrozzi, F., Capula, M. & Luiselli, L. (2013). Climate-change-related shifts in annual phenology of a temperate snake during the last 20 years. *Acta Oecologica*, **51**, 42-48.

Rzadkowska, M., Allender, M. C., O'Dell, M. & Maddox, C. (2016). Evaluation of common disinfectants effective against *Ophidiomyces ophiodiicola*, the causative agent of snake fungal disease. *Journal of Wildlife Diseases*, **52(3)**: 759-762.

Sacchi, R., Scali, S., Pellitteri-Rosa, D., Pupin, F, Gentilli, A., Tettamanti, S., Maiocchi, V., Cavigioli, L., Racina, L., Galeotti, P. & Fasola, M. & (2010). Photographic identification in reptiles: a matter of scales. *Amphibia-Reptilia*, **31(4)**, 489-502.

Saha, A., McRae, L., Dodd Jr, C. K., Gadsden, H., Hare, K. M., Lukoschek, V. & Böhm, M. (2018). Tracking global population trends: population time-series data and a living planet index for reptiles. *Journal of Herpetology*, **52(3)**, 259-268.

Sala, O. E., Chapin, F. S., Armesto, J. J., Berlow, E., Bloomfield, J., Dirzo, R., Huber-Sanwald, E., Huenneke, L. F., Jackson, R. B., Kinzig, A., Leemans, R., Lodge, D. M., Mooney, H. A., Oesterheld, M., Poff, N. L., Sykes, M. T., Walker, B. H. & Wall, D. H. (2000). Global biodiversity scenarios for the year 2100. *Science*, **287(5459)**, 1770-1774.

Sánchez-Bayo, F. & Wyckhuys, K. A. (2019). Worldwide decline of the entomofauna: A review of its drivers. *Biological Conservation*, **232**, 8-27.

Sannolo, M., Gatti, F., & Scali, S. (2014). First record of thanatosis behaviour in *Malpolon monspessulanus* (Squamata: Colubridae). *Herpetology Notes*, **7**, 323.

Santos, X., Feriche, M., León, R., Filippakopoulou, A.,Vidal-García, M., Llorente, G. A. & Pleguezuelos, J. M. (2011). Tail breakage frequency as an indicator of predation risk for the aquatic snake *Natrix maura*. *Amphibia-Reptilia*, **32(3)**, 375-383.

Sargeant, A. B. & Eberhardt, L. E. (1975). Death feigning by ducks in response to predation by red foxes (*Vulpes fulva*). *American Midland Naturalist*, **94(1)**, 108-119.

Schatz, G. E. (2009). Plants on the IUCN Red List: setting priorities to inform conservation. *Trends in Plant Science*, **14(11)**, 638-642.

Scheele, B. C., Pasmans, F., Skerratt, L. F., Berger, L., Martel, A. N., Beukema, W., Acevedo, A. A., Burrowes, P. A., Carvalho, T., Catenazzi, A., De la Riva, I., Fisher, M. C., Flechas, S. V., Foster, C. N., Frías-Álvarez, P., Garner, T. W. J., Gratwicke, B., Guayasamin, J. M., Hirschfeld, M., Kolby, J. E., Kosch, T. A., La Marca, E., Lindenmayer, D. B., Lips, K. R., Longo, A. V., Maneyro, R., McDonald, C. A., Mendelson, J., Palacios-Rodriguez, P., Parra-Olea, G., Richards-Zawacki, C. L., Rödel, M.-O., Rovito, S. M., Soto-Azat, C., Toledo, L. F., Voyles, J., Weldon, C., Whitfield, S. M., Wilkinson, M., Zamudio, K. R. & Canessa, S. (2019). Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. *Science*, **363(6434)**, 1459-1463.

Schlaepfer, M. A., Hoover, C. & Dodd, C. K. (2005). Challenges in evaluating the impact of the trade in amphibians and reptiles on wild populations. *AIBS Bulletin*, **55(3)**, 256-264.

Schwarz, C. J. (2001). The Jolly-Seber model: more than just abundance. *Journal of Agricultural, Biological, and Environmental Statistics*, **6(2)**, 195-205.

Semlitsch, R. D. (1979). The influence of temperature on ecdysis rates in snakes (genus *Natrix*)(Reptilia, Serpentes, Colubridae). *Journal of Herpetology*, **13(2)**, 212-214.

Sewell, D., Guillera-Arroita, G., Griffiths, R. A. & Beebee, T. J. (2012). When is a species declining? Optimizing survey effort to detect population changes in reptiles. *PLoS ONE*, **7(8)**, e43387.

Sewell, D., Baker, J. M. & Griffiths, R. A. (2015). Population dynamics of grass snakes (*Natrix natrix*) at a site restored for amphibian reintroduction. *The Herpetological Journal*, **25(3)**, 155-161.

Sigler, L., Hambleton, S. & Paré, J. A. (2013). Molecular characterization of reptile pathogens currently known as members of the *Chrysosporium* anamorph of *Nannizziopsis vriesii* (CANV) complex and relationship with some human-associated isolates. *Journal of Clinical Microbiology*, **51(10)**, 3338-3357.

Silvestre, A. M. (2014). How to assess stress in reptiles. *Journal of Exotic Pet Medicine*, **23(3)**, 240-243.

Smith, M. A. (1951). *British amphibians and reptiles*. London, Collins.

Smith, A. L., Gardner, M. G., Fenner, A. L. & Bull, C. M. (2009). Restricted gene flow in the endangered pygmy bluetongue lizard (*Tiliqua adelaidensis*) in a fragmented agricultural landscape. *Wildlife Research*, **36(6)**, 466-478.

Smith, M. J., Cogger, H., Tiernan, B., Maple, D., Boland, C., Napier, F., Detto, T. & Smith, P. (2012). An oceanic island reptile community under threat: the decline of reptiles on Christmas Island, Indian Ocean. *Herpetological Conservation and Biology*, **7(2)**, 206-218.

Soto-Azat, C., Clarke, B. T., Fisher, M. C., Walker, S. F. & Cunningham, A. A. (2009). Non-invasive sampling methods for the detection of *Batrachochytrium dendrobatidis* in archived amphibians. *Diseases of Aquatic Organisms*, **84(2)**, 163-166.

Soulsbury, C. D., Gray, H. E., Smith, L. M., Braithwaite, V., Cotter, S. C., Elwood, R. W., Wilkinson, A. & Collins, L. M. (2020). The welfare and ethics of research involving wild animals: A primer. *Methods in Ecology and Evolution*, **11(10)**, 1164-1181.

Spellerberg, I. F. (1972). Temperature tolerances of southeast Australian reptiles examined in relation to reptile thermoregulatory behaviour and distribution. *Oecologia*, **9(1)**, 23-46.

Spellerberg, I. F. (1975). Conservation and management of Britain's reptiles based on their ecological and behavioural requirements: a progress report. *Biological Conservation*, **7(4)**, 289-300.

Spitzen-van der Sluijs, A., Stegen, G., Bogaerts, S., Canessa, S., Steinfartz, S., Janssen, N., Bosman, W., Pasmans, F. & Martel, A. (2018). Post-epizootic salamander persistence in a disease-free refugium suggests poor dispersal ability of *Batrachochytrium salamandrivorans*. *Scientific Reports*, **8**, 3800.

Steen, D. A. (2010). Snakes in the grass: secretive natural histories defy both conventional and progressive statistics. *Herpetological Conservation and Biology*, **5(2)**, 183-188.

Steffen, W., Grinevald, J., Crutzen, P. & McNeill, J. (2011). The Anthropocene: conceptual and historical perspectives. *Philosophical Transactions of the Royal Society A*: *Mathematical, Physical and Engineering Sciences*, **369(1938)**, 842-867.

Stegen, G., Pasmans, F., Schmidt, B. R., Rouffaer, L. O., Van Praet, S., Schaub, M., Canessa, S., Laudelout, A., Kinet, T., Adriaensen, C., Haesebrouck, F., Bert, W., Bossuyt, F. & Martel, A. (2017). Drivers of salamander extirpation mediated by *Batrachochytrium salamandrivorans*. *Nature*, **544(7650)**, 353-356.

Steward, J.W. (1971). *The Snakes of Europe*. David & Charles, Exeter, UK, Sun, P. L., Yang, C. K., Li, W. T., Lai, W. Y., Fan, Y. C., Huang, H. C. & Yu, P. H. (2021). Infection with *Nannizziopsis guarroi* and *Ophidiomyces ophiodiicola* in reptiles in Taiwan. *Transboundary and Emerging Diseases*, **69(2)**, 764-775.

Suriyamongkol, T., & Mali, I. (2018). Feasibility of using computer-assisted software for recognizing individual Rio Grande Cooter (*Pseudemys gorzugi*). *Copeia*, **106(4)**, 646-651.

Takami, Y., Nam, K. O., Takaki, Y., Kadekaru, S., Hemmi, C., Hosoya, T. & Une, Y. (2021). First report of ophidiomycosis in Asia caused by *Ophidiomyces ophiodiicola* in captive snakes in Japan. *Journal of Veterinary Medical Science*, **83**, 1234-1239.

Tapley, B., Griffiths, R. A. & Bride, I. (2011). Dynamics of the trade in reptiles and amphibians within the United Kingdom over a ten-year period. *The Herpetological Journal*, **21(1)**, 27-34.

Tetzlaff, S. J., Ravesi, M. J., Allender, M. C., Carter, E. T., DeGregorio, B. A., Josimovich, J. M. & Kingsbury, B. A. (2017). Snake fungal disease affects behavior of free-ranging massasauga rattlesnakes (*Sistrurus catenatus*). *Herpetological Conservation and Biology*, **12(3)**, 624-634.

Tilman, D., Clark, M., Williams, D. R., Kimmel, K., Polasky, S. & Packer, C. (2017). Future threats to biodiversity and pathways to their prevention. *Nature*, **546(7656)**, 73-81.

Thomas, R. A. & Hendricks F. S. (1976). Letisimulation in *Virginia striatula* (Linnaeus). *The Southwestern Naturalist*, **21(1)**, 123-124.

Thomas, B., Holland, J. D. & Minot, E. O. (2011). Wildlife tracking technology options and cost considerations. *Wildlife Research*, **38(8)**, 653-663.

Thorpe, R. S. (1989). Pattern and function of sexual dimorphism: a biometric study of character variation in the grass snake (*Natrix natrix*, Colubridae) due to sex and its interaction with geography. *Copeia*, **1989(1)**, 53-63.

Toledo, L. F., Sazima, I. & Haddad, C. F. (2011). Behavioural defences of anurans: an overview. *Ethology Ecology & Evolution*, **23(1)**, 1-25.

Tozetti, A. M. & Martins, M. (2007). A technique for external radio-transmitter attachment and the use of thread-bobbins for studying snake movements. *South American Journal of Herpetology*, **2(3)**, 184-190.

Tryon B. W. & Guese R. K. (1984). Death-feigning in the gray-banded kingsnake *Lampropeltis alterna*, *Herpetological Review* **15(4)**, 108-109.

Turner, R. K., Griffiths, R. A., Wilkinson, J. W., Julian, A. M., Toms, M. P. & Isaac, N. J. (2022). Diversity, fragmentation, and connectivity across the UK amphibian and reptile data management landscape. *Biodiversity and Conservation*. Advance online publication.

Veilleux, J., Dombrowski, D. S., Allender, M. C. & Lewbart, G. (2020). Diagnosis, treatment and post-release monitoring of an eastern black rat snake (*Pantherophis alleghaniensis*) with ophidiomycosis and traumatic injuries. *Veterinary Record Case Reports*, **8(2)**, e000954.

Vijay, V., Pimm, S. L., Jenkins, C. N. & Smith, S. J. (2016). The impacts of oil palm on recent deforestation and biodiversity loss. *PLoS ONE*, **11(7)**, e0159668.

Uetz, P., Freed, P, Aguilar, R. & Hošek, J. (eds.) (2022) The Reptile Database, http://www.reptile-database.org, accessed [21st September 2022].

Urban, M. C., Richardson, J. L., & Freidenfelds, N. A. (2014). Plasticity and genetic adaptation mediate amphibian and reptile responses to climate change. *Evolutionary Applications*, **7(1)**, 88-103.

Wagner, N., Mingo, V., Schulte, U. & Lötters, S. (2015). Risk evaluation of pesticide use to protected European reptile species. *Biological Conservation*, **191**, 667-673.

Ward, R. J., Griffiths, R. A., Wilkinson, J. W. & Cornish, N. (2017). Optimising monitoring efforts for secretive snakes: a comparison of occupancy and N-mixture models for assessment of population status. *Scientific Reports*, **7**, 18074.

Wake, D. B. & Vredenburg, V. T. (2008). Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proceedings of the National Academy of Sciences*, **105**, 11466–11473.

Webb, J. K., Brook, B. W. & Shine, R. (2002). What makes a species vulnerable to extinction? Comparative life-history traits of two sympatric snakes. *Ecological Research*, **17(1)**, 59-67.

Wiles, G. J., Bart, J., Beck Jr, R. E. & Aguon, C. F. (2003). Impacts of the brown tree snake: patterns of decline and species persistence in Guam's avifauna. *Conservation Biology*, **17(5)**, 1350-1360.

Willson, J. D. (2016). ‘Surface-dwelling reptiles’ in Dodd, C. K. (Ed.) *Reptile Ecology and Conservation: A Handbook of Techniques*. Oxford University Press, UK, 125-138.

Wilson, R. P., Börger, L., Holton, M. D., Scantlebury, D. M., Gómez-Laich, A., Quintana, F., Rosell, F., Graf, P. M., Williams, H., Gunner, R., Hopkins, L., Marks, N., Geraldi, N. R., Duarte, C. M., Scott, R., Strano, M. S., Robotka, H., Eizaguirre., C., Fahlman., A. & Shepard, E. L. C. (2020). Estimates for energy expenditure in free-living animals using acceleration proxies: A reappraisal. *Journal of Animal Ecology*, **89(1)**, 161-172.

Wintle, B. A., Kavanagh, R. P., McCarthy, M. A. & Burgman, M. A. (2005). Estimating and dealing with detectability in occupancy surveys for forest owls and arboreal marsupials. *The Journal of Wildlife Management*, **69(3)**, 905-917.

Woodburn, D. B., Miller, A. N., Allender, M. C., Maddox, C. W. & Terio, K. A. (2019). *Emydomyces testavorans*, a new genus and species of onygenalean fungus isolated from shell lesions of freshwater aquatic turtles. *Journal of Clinical Microbiology*, **57(2)**, e00628-18.

Woodfine, T., Wilkie, M., Gardner, R., Edgar, P., Moulton, N. & Riordan, P. (2017). Outcomes and lessons from a quarter of a century of Sand lizard *Lacerta agilis* reintroductions in southern England. *International Zoo Yearbook*, **51(1)**, 87-96.

Wylie, G. D., Smith, J. J., Amarello, M. & Cassazza, M. L. (2011). A taping method for external transmitter attachment on aquatic snakes. *Herpetological Review*, **42(2)**, 187.

Yang, J. H. & Chan, B. P. L. (2015). Two new species of the genus *Goniurosaurus* (Squamata: Sauria: Eublepharidae) from southern China. *Zootaxa*, **3980(1)**, 67-80.

Yoccoz, N. G., Nichols, J. D. & Boulinier, T. (2001). Monitoring of biological diversity in space and time. *Trends in Ecology & Evolution*, **16(8)**, 446-453.

Yu, S., Halbrook, R. S., Sparling, D. W. & Colombo, R. (2011). Metal accumulation and evaluation of effects in a freshwater turtle. *Ecotoxicology*, **20(8)**, 1801-1812.

Zhang, J., Finlaison, D. S., Frost, M. J., Gestier, S., Gu, X., Hall, J., Jenkins, C., Parrish, K., Read, A. J., Srivastava, M., Rose, K. & Kirkland, P. D. (2018). Identification of a novel nidovirus as a potential cause of large scale mortalities in the endangered Bellinger River snapping turtle (*Myuchelys georgesi*). PLoS ONE, **13(10)**, e0205209.

Zipkin, E. F., DiRenzo, G. V., Ray, J. M., Rossman, S. & Lips, K. R. (2020). Tropical snake diversity collapses after widespread amphibian loss. *Science*, **367(6479)**, 814-816.

Zylstra, E. R., Steidl, R. J., Jones, C. A. & Averill-Murray, R. C. (2013). Spatial and temporal variation in survival of a rare reptile: a 22-year study of Sonoran desert tortoises. *Oecologia*, **173(1)**, 107-116.