THE POWER OF METABARCODING FOR INVESTIGATING THE TROPIC INTERACTIONS OF MESOPREDATORS THROUGH DIETARY STUDIES.

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Abstract

Diet studies can contribute knowledge about the ecology of a species, its interactions with other species and information about the wider environment. They are therefore important tools in effective conservation planning. Studying the diet of mesopredators is of increasing importance due to the widespread loss of apex predators, resulting in the dominance of the smaller predators. Despite the increasing importance of mesopredators in ecosystems, they remain understudied relative to larger more charismatic apex species. Recent advances in the field of metabarcoding has led to a renaissance in the number of diet studies being undertaken due to the increased ability to identify prey items, especially small items or those that little or no identifiable remains for more traditional morphological methods to assess. Metabarcoding is capable of processing hundreds or even thousands of samples simultaneously, presenting opportunities to conduct large-scale studies with far more powerful conclusions than the smaller scale studies commonly conducted in the past. Although metabarcoding has become a powerful tool, it suffers from a wide variety of biases that need to be accounted for, and doing so has increased the size, complexity and cost of metabarcoding studies. One way of conducting large scale studies cost-effectively is to use a tagged barcode approach to create metabarcoding libraries. While this approach is cost effective, it needs a wide variety of specialist equipment to prepare the libraries for sequencing, making it difficult for some smaller laboratories to execute such studies. In this thesis, I developed a library preparation protocol that could be used in the absence of some of the quality control equipment commonly recommended in all tag-based library preparation techniques, thereby increasing the accessibility of metabarcoding work to a range of laboratories. I validated this method showing it produced precise mock communities, with a good level of accuracy. This methodology was then validated using a novel hierarchical marker strategy to assess the full breadth of the diet of two mesocarnivores, Neotropical and Eurasian Otters, more effectively than previous studies. I found that invertebrates represented a significant portion of otter diets, something previously assessed. Metabarcoding diet also revealed wider changes in the environment, including the spread of invasive species outside of their reported range. The methodology also revealed the previously unknown importance of plants and scavenging behaviour to the critically

endangered Bermuda skink, informing both in-situ and ex-situ conservation plans. Overall, this work demonstrated the role that cost-effective metabarcoding methodology can contribute to ecological and conservation understanding.

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Chapter 1 – Thesis introduction

Diet studies and their importance for conservation

Predators play a fundamental role in ecosystems with relatively few species controlling the population sizes of many within their communities (Ritchie *et al.*, 2012). Large apex predators have received the most attention in this regard with many studies focusing on their effects on community and ecosystem dynamics (Roemer, Gompper and Van Valkenburgh, 2009; Ray *et al.*, 2013). Despite this, smaller mesopredators are larger in number and have an important impact on the communities they inhabit but remain relatively understudied relative to apex predators (Roemer, Gompper and Van Valkenburgh, 2009). This is an oversight as the loss of large predators has left mesopredators as de facto apex predators with greater power to shape their communities, therefore further studies looking into the ecological role of mesopredators are necessary for understanding current and future ecosystem dynamics (Ritchie and Johnson, 2009).

In order to gain a full understanding of ecosystem function, it is essential to uncover the trophic interactions and food webs that it contains (de Sousa, Silva and Xavier, 2019). The knowledge of species interactions allow us to understand species competition, predation pressure and other pressures species may be under (Mitchell and Banks, 2005). Dietary studies can reveal the trophic position of species and the interactions of those species through consumption, thereby unveiling the impact one species has on another within their community (Pauly *et al.*, 1998; de Sousa, Silva and Xavier, 2019). Consumer and prey interactions underpin the majority of the interactions within food webs and the understanding of these interactions are necessary if ecological management plans are to be realistic and successful (Bascompte and Melián, 2005). Such pressures are not limited to solely carnivores. Many smaller predators are omnivorous and therefore create predation and competition pressures, not just on other animal species but on plant communities (Kratina *et al.*, 2012). The unprecedented rate of change the earth is currently undergoing is changing community structure and the trophic positions species occupy. Understanding how these trophic interactions are changing such, as by

investigating predator-prey dynamics, not only as they are but monitoring them as they undergo changes, is essential for conservation (Zhang *et al.*, 2017).

Despite the importance in understanding consumer-resource interactions of mesopredators, such information has always been limited due to the complexity in accurately identifying the wide variety of food items they consume (Jakubavičiute *et al.*, 2017; Kurita *et al.*, 2022). This is particularly true of omnivorous species; such species often display a high degree of plasticity in their consumption of prey, depending on many factors including season, nutritional needs and even individual diet preferences (Tercel, Symondson and Cuff, 2021). Historically, methods used to analyse diet have included direct observation of behaviour, microscopic analysis, stable isotope analysis, electrophoresis of gut contents, and protein electrophoresis, amongst others (da Silva *et al.*, 2019). Although all these techniques have their own unique technical limitations, the limitation they all share is that they are unable to investigate the full spectrum of the diet displayed by many omnivorous and mesopredators (Deiner *et al.*, 2017). More recently, metabarcoding as become a popular method that can investigate the full breadth of such diets. By utilising the power of next generation sequencing, it is possible to sequence hundreds of samples simultaneously and cost effectively (Cilleros *et al.*, 2019). Through the use of multiple "universal barcodes", short fragments of DNA that vary within a certain taxa, species present within samples can be identified accurately and efficiently. Employing multiple barcodes makes it possible to identify prey species from all aspects of the diet of a predator and draw more powerful conclusions than was possible prior to the advent of metabarcoding (Taberlet *et al.*, 2018).

Metabarcoding for studying diet

Metabarcoding has become a well-recognised tool for studying species diet due to the advantages of high taxonomic resolution that it confers over other methodologies (Thomsen and Willerslev, 2015; Taberlet *et al.*, 2018). This is especially true with species that were difficult to study with previously available techniques due to their dietary items being too small or difficult to identify as they are too digested and/or lack identifiable parts (Monterroso *et al.*, 2019). This is especially true of dietary studies based on morphologically identifying prey items in scat or though identifying remains

though partially digested stomach contents obtained via autopsy, such studies account for the vast majority of diet studies prior to metabarcoding, these studies however are often limited in scale due to the time consuming nature of identifying all recognisable remains in each scat obtained. Metabarcoding has also allowed the study of species that have until now been unstudied due to their elusive behaviour making direct observation of feeding or recovery of scat difficult or practically impossible (Ingala *et al.*, 2021; Brun *et al.*, 2022). The power of metabarcoding has led to a renaissance in diet studies. Despite this increase in the power, there are significant technical issues that can introduce significant bias into the data produced by metabarcoding of diet, requiring increasingly complex experimental designs to account for them (Zinger, Bonin, Inger G. Alsos, *et al.*, 2019).

The aims of diet studies using metabarcoding generally are to uncover dietary richness, composition, and abundance (Ando *et al.*, 2020). These measures of the diet are used as a proxy for the energetic importance of the dietary components identified. Despite the nutritional contribution of prey species to the predator being a central goal for all metabarcoding studies, there remains a high degree of uncertainty about the accuracy of the data produced (Cuff *et al.*, 2022). Metabarcoding can introduce error into datasets at many stages. It can often overinflate species richness due to the introduction of errors in PCR, while the universal barcodes used to identify species are imperfect leading to both the over and underestimation of certain taxa in the data (Pollock *et al.*, 2018; Taberlet *et al.*, 2018; Forstchen, 2020). Finally, contamination is an inherent risk in metabarcoding studies and necessitates careful experimental design to ensure accuracy (Forstchen, 2020). The degree to which the above biases impede our ability to make inferences about abundances, composition and species richness remains to be fully resolved, with designs becoming increasingly complex and expensive as new issues are discovered (Schnell, Bohmann and Gilbert, 2015; Pollock *et al.*, 2018; Forstchen, 2020).

Metabarcoding is expensive by its nature, and it requires a large amount of specialist equipment to implement. In many parts of the world this equipment is either unavailable or more difficult to get hold of than it is in the areas where these methods are being developed. The increase in our understanding of the biases present in metabarcoding studies has created the need for more complex and expensive experimental designs (Taberlet *et al.*, 2018; Zinger, Bonin, Inger G. Alsos, *et al.*, 2019; Ando *et al.*, 2020). Although the need for this experimental robustness is necessary, the constant increase in

cost and complexity risks placing this valuable and important tool out of reach of many laboratories around the world. Creating methodologies that are accessible to a greater proportion of laboratories, while maintaining the experimental robustness required, is of great importance for conservation globally. Many nations with the greatest biodiversity are those that are currently undergoing the highest rates of land use change and biodiversity loss, and they also lack the resources to undertake the research required to assess and conserve their biodiversity (WWF, 2020). Therefore, increasing the accessibility of metabarcoding may help conservation efforts in many parts of the world helping us study ecosystem dynamics more effectively (Cilleros *et al.*, 2019; Ruppert, Kline and Rahman, 2019).

The creation or development of a library preparation method that allows laboratories with less capital equipment to undertake metabarcoding experiments is a priority. Library preparation can be one of the most expensive steps in a metabarcoding approaches, both in reagent cost and considerable investment in equipment such as qPCR machines and bioanalyzers, which are expensive and in some areas unavailable (Bohmann *et al.*, 2021). A cheaper but robust preparation method that negates some of the need for such equipment would be extremely useful for many laboratories.

The development of a cost-effective library preparation method will also allow the study of species with broad diets to be investigated more effectively (da Silva *et al.*, 2019; Tercel, Symondson and Cuff, 2021). Species with broad diets are complex to study due to the cost of multiple marker approaches needed to assess all aspects of their diet. A species that is omnivorous, as many mesopredators are, or even species that are carnivores but eat a wide variety of prey such as insects and vertebrates, require multiple markers to explore such diets effectively (M. De Barba *et al.*, 2014; Tercel, Symondson and Cuff, 2021; Topstad *et al.*, 2021). The cost of metabarcoding goes up proportionately to the number of markers used, and this is true of the more popular library preparation methods such as two-step PCR (Bohmann *et al.*, 2021). The adoption of tag-based strategies promises to make the library preparation only fractionally more expensive with multiple marker approaches, making the study of these increasingly important groups more realistic targets for future research.

The technical approaches to metabarcoding

Here we outline some of the common approaches used for the production of metabarcoding libraries and the issues faced by all of the common approaches, as well as specific issues associated with each technique.

All metabarcoding experiments need library preparation and sequencing (Taberlet *et al.*, 2018; Liu *et al.*, 2020a). All of these stages can introduce bias or contamination to samples, and therefore need to be considered carefully in order to draw valid ecological conclusions from the samples collected (Alberdi *et al.*, 2018; Bohmann *et al.*, 2021). In order to control for the various sources of bias, experimental designs are becoming increasingly complex, making the planning and execution of metabarcoding experiments more expensive and time consuming (Schnell, Bohmann and Gilbert, 2015; Zinger, Bonin, Inger G. Alsos, *et al.*, 2019). By far the most popular platform for metabarcoding is the Illumina sequencing series, due to their high output accuracy and low per base cost (Bohmann *et al.*, 2022). The strength of metabarcoding approaches is the ability to utilise the enormous capacity of high throughput sequencing platforms to sequence hundreds or even thousands of samples simultaneously (Thomsen and Willerslev, 2015; Huggett *et al.*, 2017). This is achieved by tagging samples with a unique barcode that allows the origin of a sequence to be determined after all samples have been pooled and placed on the sequencer simultaneously for sequencing . These barcodes can be introduced to the samples at two sample preparation stages: in the initial PCR amplification with barcodes being present on the universal barcode primers, or later in the library preparation stage. Although there are many unique metabarcoding lab approaches, they fall into three broad categories; One-step PCR, Two-step PCR and tagged PCR methodologies (Zizka *et al.*, 2019a; Bohmann *et al.*, 2021). Each of these strategies comes with compromises that need to be accounted for in any downstream analysis of the data produced.

One-step PCR approaches have universal barcode primers that contain not only the primer sequence for the metabarcode but also the identifying barcode and the adapter sequence that binds the DNA to the sequencer (Zizka *et al.*, 2019a). These primers are known as “fusion primers”. Fusion primers allow the samples to be pooled and normalized for sequencing directly after the initial PCR. The appeal of this approach is its simplicity; after the initial PCR, the samples are uniquely tagged and amplified meaning they can be pooled, cleaned, and normalised for sequencing immediately, which

simplifies the library preparation as well as reducing the cost of that stage. However, such large primers often reduce amplification efficiency because due to their large size they have decreased affinity for the target region and bind less effectively. This often dramatically changes community composition. Such fusion primers are also very expensive due to their size and the high degree of purification needed in order for them to be compatible with sequencers. The high cost of these primers often outweighs the savings in the library preparation stage and has led to this method falling in popularity in recent years (Zizka *et al.*, 2019b).

A two-step PCR approach is currently the most common strategy for metabarcoding (Head *et al.*, 2014; Liu *et al.*, 2020a). This method and relies on two PCR phases, the first to amplify samples and the second to uniquely label each sample. The first PCR with this approach amplifies the sample with a universal primer that contains an adapter "tail". Subsequently, a second PCR adds the adapter sequences to the initial metabarcodes using a complimentary sequence to the tail present on the first set of primers. This method has been found to be a robust and reliable method, which accounts for its popularity (Lampa *et al.*, 2008; Pjevac *et al.*, 2021). However, there are several issues associated with the two-step approach that have led to the development of other methods. As the samples are only uniquely identifiable after the second PCR, each sample has to be library-prepped separately meaning that large experimental designs containing hundreds, or thousands of samples quickly become time consuming and expensive. Although automated approaches may solve the time-related issues, the expense involved in library prepping so many samples are intrinsic to the method. Furthermore, the increased number of PCR cycles can affect the proportions of species present in samples and increase chimera production (Krehenwinkel *et al.*, 2017). Despite these limitations, the two-step approach has been shown to produce reliable data if these limitations are accounted for.

A tagged PCR approach amplifies samples with a universal barcode that contains a small unique "tag" usually between 6 to 10bp. Each sample contains either a unique tag or unique combination of these tags (Pompanon *et al.*, 2012; Taberlet *et al.*, 2018). These samples can then be pooled after the initial PCR step as is the case in the one-step strategy. These pools can then be library prepped in one tube (Carøe and Bohmann, 2020). The library preparation is usually undertaken with ligation as opposed to PCR as was the case with a two-step strategy. The ligation methodology keeps the PCR cycles to a minimum thus preserving the community composition as accurately as possible. The

primers are comparatively cheap relative to fusion primers, and pooling prior library preparation makes this method much cheaper for large datasets and reduces the complexity of the lab-work, reducing error and making working with large and complex datasets easier. However, the pools of samples are more susceptible to inter-sample contamination than other methodologies due to issue such as tag-jump, which can account for up to 49% of the data produced (Carøe and Bohmann, 2020). However, these tag jumps can be kept to manageable levels if experimental design is robust (Hamback, Sargac and Grudzinska-Sterno, 2022).

Researchers are increasingly using tagged approaches to library preparation the cost savings outweigh the potential issues of tag jump which can be controlled while the decreased cost allows for more samples, replicates, and controls increasing the accuracy of the data produced. Despite this saving the need for expensive equipment to validate such libraries remains a barrier and the development of alternative validation methods in the absence of such equipment will be of value to many laboratories.

Biases inherent in metabarcoding library preparation

Contamination

Contamination can be introduced into metabarcoding data at all stages of the process, and it is imperative to the success of any project to keep the contamination to the lowest possible level, the main ways in which bias can be introduced at the library preparation stage is the changing of community composition through increased PCR cycling and production of chimeras (Zinger, Bonin, Inger G. Alsos, *et al.*, 2019; Bohmann *et al.*, 2022). There is also a risk of cross contamination of samples through tag-jump potentially inflating alpha diversity in samples and drowning any signal with increased noise (Zinger *et al.*, 2021).

Chimeras

Chimeras are a significant issue with metabarcoding and are produced during PCR amplification (Bjørnsgaard Aas, Davey and Kauserud, 2017). The rate of chimera

production is affected by the PCR conditions, with a short elongation phase leading to incomplete fragments that can then interact with other DNA fragments leading to an inflation of species richness (Schnell, Bohmann and Gilbert, 2015). The number of PCR cycles is also important with a higher number of cycles often increasing chimera production as in the later cycles primers can be exhausted leading to incomplete products acting as primers and creating chimeras in large numbers. The stage of library preparation the chimeras are produced in is important. If they occur in the first stage of amplification then they will increase the alpha diversity present in the sample artificially (Pauvert *et al.*, 2019). If chimera production occurs in later phases when samples are pooled, it can also lead to inter-sample contamination that can artificially inflate both alpha and beta diversity across the dataset.

Tag-jump

Finally, another source of contamination is tag-jump, which is a form of inter-sample contamination introduced with ligation-based library preparation methods that pool samples prior to library preparation (Schnell, Bohmann and Gilbert, 2015; Carøe and Bohmann, 2020). When samples are tagged with a unique barcode combination in the initial PCR stage then all samples can be pooled, which reduces cost. However, the reduction in financial cost comes with the increased risk of cross contamination. Most ligation-based methodologies include a blunt ending step to ensure the adapters required for binding the DNA sequence to the flowcell are ligated efficiently (Leray, Haenel and Bourlat, 2016; Zizka *et al.*, 2019a). This process involves repairing the ends of the DNA sequence that often includes the barcode. If the barcode on the forward and reverse primer are different, then the blunt-ending enzyme will use the complementary sequence as a template for the repair, which leads to the repaired strand containing the wrong barcode, and thus potentially assigning that sequence to a different sample and by inference, potentially introducing a species to a sample that was not truly present (Alberdi *et al.*, 2018). The reported rates of tag jump varies widely but can range from less than one percent to 49%, thus making it a major factor to consider with tagged metabarcoding strategies (Carøe and Bohmann, 2020).

Accounting for bias in metabarcoding

The wide range of potential biases that metabarcoding can introduce into a dataset requires a robust experimental design to effectively account for them (Zinger, Bonin, Inger G Alsos, *et al.*, 2019). Positive controls are essential in determining the accuracy of abundance data and should be included in all experiments (Taberlet *et al.*, 2018). These can be used to assess the effectiveness of a marker and any bias it may have introduced to the dataset. Internal spike-ins have been suggested to control for biases within samples and to reduce the noise present in datasets, making conclusions drawn from them more robust (Harrison *et al.*, 2021). Extraction negative controls are essential as are PCR negatives. These reduce the impact of contamination from these stages and identify if contamination has compromised an experiment (Zinger, Bonin, Inger G. Alsos, *et al.*, 2019; Forstchen, 2020). Finally, sequencing controls are essential, especially if using a strategy that pools samples prior to or during library preparation, as these methods can introduce high levels of inters ample contamination and the presence of these controls allows for the data to be bioinformatically cleaned so such inter-sample contamination can be reduced or removed from datasets (Schnell, Bohmann and Gilbert, 2015). Finally, technical replicates must be included as the stochastic variation introduced by PCR especially in samples with low DNA concentrations can led to very different communities being amplified from the same sample (Liu *et al.*, 2020a). Ideally the number of technical replicates should be determined experimentally, but a minimum of three is a generally accepted number for most experiments. Although the inclusion of all these controls and replicates greatly increases the complexity and work needed to conduct an metabarcoding study, they are essential if the results of a study are to be robust and yield results of any value.

Aims of the thesis

To resolve a current gap in the preparation of tagged-based library preparation strategies. Although such approaches have been researched extensively (Taberlet *et al.*, 2018; Carøe and Bohmann, 2020), such efforts have focused on the production of techniques that reduce a specific limitation such as tag jump levels. Although this is important, the cost and, in some cases, lack of equipment used in such protocols can prevent smaller

laboratories from attempting such projects. Here, we demonstrate a tagged strategy that uses the commercial kit sold by Illumina wherever they sell sequencers and needs minimal specialist quality control equipment to produce robust and effective sequencing libraries (Chapter 2). We then use this to test a novel marker strategy that will allow for the quantification of the importance of different parts of mesopredator diets, increasing our knowledge of this important group of predators. We tested this strategy first on a well-studied mesocarnivore, the Eurasian Otter *Lutra lutra,* as it is easier to assess the accuracy of results with a well-studied species (Chapter 4), first conducting a literature review to ensure chose our markers correctly (Chapter 3). We then used our strategy on a less well studied but related species, the Neotropical Otter *Lontra longicaudis,* to demonstrate this method can be used to explore novel environments (Chapter 5). Finally, we performed an exploratory study on a mesopredator of which we knew almost nothing about the diet, the Bermuda skink *Plestiodon longirostris*, to help improve conservation efforts for this critically endangered species (Chapter 6).

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Chapter 7 General Discussion

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Chapter 2 – A commercial kit-based ligation methodology for metabarcoding with low per sample cost and with limited access to equipment

Abstract

Metabarcoding has become an essential tool for investigating biodiversity and has been increasingly adopted to answer a wide variety of ecological questions that assess abundance and frequency of species within and between systems. Despite its increasing popularity, there is awareness of its susceptibilities to biases has led to increasingly complex and therefore costly experimental designs. Preparing libraries can be costly and the quality control stages of library preparation techniques often require specialist equipment that may not be available. Here we present a tag based library preparation methodology that is low cost, highly precise (greater than 95%) while keeping internal contamination low (between 2-7% on average). This was confirmed using a known mock community using three different markers (Eukaryotic, Vertebrate and Invertebrate) to assess precision and accuracy of our method. The levels of precision were obtained by evaluating the variance in seven experimental treatment groups. Our mock community compositions varied significantly from the theoretical distributions in line with precious investigations on metabarcoding which indicate abundance is to be treated with caution. We therefore recommend our method as a low-cost practical solution for laboratories that may lack some specialist equipment and or access to the reagents to currently undertake best practice metabarcoding.

Introduction

Investigating the diversity of organisms present in ecosystems through the amplification of barcoding regions of DNA has become an increasingly important tool across several fields, and has been instrumental in reshaping our views on the determination of

evolutionary history, taxonomy, and diversity in many environments (Shoba Ranganathan *et al.*, 2019). Prior to the advent of high throughput sequencing, the cost and difficulty in sequencing a wide range of species in any given environment was unfeasible, but the arrival of new sequencing chemistries, in particular Illumina, has reduced costs more than 10 fold, while making it possible to multiplex thousands of highly complex samples simultaneously (Thomsen and Willerslev, 2015). In order to multiplex large numbers of samples, many 'tagging' strategies have been developed that mark each sample with a unique identifying barcode that allows all sequences to be pooled without losing track of the originating sample (Bowers *et al.*, 2015; Bohmann *et al.*, 2021). The most common approach currently is a two-step reaction protocol where each sample is amplified with primers that have a universal tail of some description and subsequently a second PCR reaction is performed where adapter sequences are added that also contain unique barcodes for each individual sample (Liu *et al.*, 2020a). These adapted and barcoded samples can then be pooled and placed on the sequencer. Another method is a ligation based one step PCR methodology where sequences are amplified with primers that contain unique identifying tags within the primer sequences themselves. These tags can either be the same on the forward or reverse primer or can be different (Zizka *et al.*, 2019a). Both one step and two step strategies have enabled advances in our understanding but have also shown themselves to be vulnerable to several forms of contamination (e.g. cross contamination, tag jump) requiring the development of more robust but consequently more expensive experimental setups.

In order for metabarcoding to be an effective tool in the characterisation of biodiversity it needs to produce accurate and reliable results. There are however, several stages at which biases can be introduced into datasets that could undermine the validity of any metabarcoding study (Pollock *et al.*, 2018). The first and most widely studied is contamination. This can occur at any stage of the study from sample collection through to extraction, PCR and ultimately sequencing. This has been thoroughly documented and there are a number of negative control types that can be used to identify and compensate for such occurrences (Forstchen, 2020). The second source of contamination is chimeric products formed from PCR reactions or subsequent library preparation steps (Schnell, Bohmann and Gilbert, 2015). The final form of contamination is erroneous sample assignment that can be caused by tag jump and has

been shown to be a considerable obstacle to producing reliable data (O'Rourke *et al.*, 2020). As a result of all these potential pitfalls designing a robust metabarcoding project is not simple and a low cost method for preparing libraries is essential if all the technical replicates and controls suggested to be included by the literature are to be realistically included (Zinger, Bonin, Inger G Alsos, *et al.*, 2019).

Despite the fact all metabarcoding studies must undergo library preparation there has until recently, been scant attention paid to biases introduced during library preparation. The potential for library preparation methodologies to impact the makeup of sample communities relative to other stages of the metabarcoding process remains underexplored (Head *et al.*, 2014). There are several stages in the preparation of NGS libraries that can introduce bias into the resulting data. The first or second PCR step can introduce chimeric products (Alberdi *et al.*, 2018) and if samples have been pooled prior to the PCR, there is the possibility of tag jump where the tag combination of one sample is switched to the tag combination of another. In post PCR pooling the rate of tag switching can reach as high as 23% (Zizka *et al.*, 2019a). The most likely part of library preparation methods to introduce tag jump is the blunt-ending step in protocols. This is the case in almost all ligation protocols, and if different tags are included on the forward and reverse sequence, the blunt-ending enzyme can incorrectly add the tag from the forward end of the sequence to the reverse of the other or vice versa, potentially causing misidentification (Schnell, Bohmann and Gilbert, 2015; Carøe and Bohmann, 2020). Currently most ligation-based methods include a blunt ending step and therefore testing which of the current methodologies induces the most bias, and in what ways, is important so we can account for the biases and reconstruct accurate communities from the samples we collect.

Currently the most common methodology utilised in metabarcoding studies is a two-step approach, which broadly encompasses several well cited protocols that include two PCR steps (Carøe and Bohmann, 2020; Bohmann *et al.*, 2021). Despite being a robust and effective method for producing indexed libraries, two-step methodologies increase the number of PCR cycles that each sample undergoes and this increases the chances of generating spurious products, and increases the degree of amplification bias in the data, making abundance estimates less reliable (Krehenwinkel *et al.*, 2017; Fonseca, 2018).

Furthermore, as each sample can only be pooled after the second PCR, as this is the stage at which the unique identifying sequences are added to the amplicons, each sample must be prepared individually thus increasing cost and the time required to prepare the libraries (Bohmann *et al.*, 2021). This often leads to a reduced number of samples, technical replicates, or controls in studies in order to keep costs to a reasonable level (Pjevac *et al.*, 2021). An increasingly popular approach is a one-step tagged PCR method where each sample is assigned a unique tag combination in the first PCR. These tags are included on the primers and can either be the same or different on the forward and reverse primer (Leray, Haenel and Bourlat, 2016). Once the first round of PCR has been completed, the samples can be pooled and the adapter sequences ligated onto the pooled library. This allows potentially thousands of samples to be prepped in just a few pools, vastly reducing the time and cost of library perpetration per sample, and allowing for increasingly large studies with more robust experimental designs, albeit at the potential cost of increased tag jump (Shirazi, Meyer and Shapiro, 2021).

The inclusion of multiple types of positive and negative controls as well as technical replicates in order to produce robust results has made undertaking metabarcoding projects increasingly expensive, although this reality has not really been acknowledged in the literature. This has placed metabarcoding further out of reach of many labs with limited funding or in areas where supplies of reagents or equipment are not readily available. This is ironic as these limitations exist in areas with the highest biological diversity and are currently undergoing the highest rates of habitat loss and urbanisation, and therefore, should be at the forefront of metabarcoding as it has the potential to rapidly record and monitor the diversity present in threatened ecosystems. Developing robust protocols that can keep costs to a minimum while retaining the experimental robustness promoted in recent years, is clearly an important steppingstone for conservation ecology in these areas.

Here we present a method based on the Truseq PCR free protocol that can produce highly consistent results with tolerable levels of chimera production and tag jump while keeping the library preparation costs as low as possible. We alter several of the steps included in the standard method including altered different bead clean-up steps based

on marker length and alternative quality control steps if institutions lack certain high cost machines opening the method to a wide variety of laboratories otherwise excluded from this approach. Each library could contain over 1000 samples, while requiring only some of the quality control equipment recommended by Illumina and others. This will allow institutions that currently struggle to undertake such projects to produce high quality research in this field. To validate our protocol, we created a mock community of 16 species and tested it with eukaryotic, vertebrate and invertebrate markers. The markers were chosen as they are widely used in metabarcoding and have been recommended to preserve abundance data more consistently than many markers due to the highly conserved nature of their binding sites leading to lopwer amplification biases. Reducing amplification bias is important to this investigation as we wish to investigate the impact of the library preparation method on abundance as well as its ability of identify all species present therefore reducing upstream bias was important. We investigate the accuracy and precision of this method in reconstructing the theoretical distribution of the mock community, while also investigating levels chimera production, tag jump and other biases in the data and ultimately look at how accurately the original communities could be reconstructed from the post sequencing data.

Methods

Mock community design

We created a mock community consisting of 16 invertebrate and vertebrate species from across the eukaryotic phylogeny (Table 2.1). DNA was extracted from either fresh or frozen tissue samples of known origin (Table 2.1). The concentration of species' DNA differed across a log2 scale (Table 2.1). DNA was quantified using Qubit and pooled in the predetermined ratio of concentrations. The species chosen were amplified individually to ensure quality of DNA. The PCR conditions were then optimised using a qPCR for each marker to ensure an optimal cycle number was chosen reducing the chances of chimera production.

Actual mock communities were embedded within runs for three other studies (on *Lutra lutra, Lontra longicaudis* and Bermudan skink) where they acted as positive controls. We

used a design based on that outlined by Taberlet *et al*. (2018). Each sample was split into technical replicates and were arranged randomly within a matrix that consisted of four 96 well plates. Random assignment to plates was stratified so that in each plate there were five positive controls, five negative controls. Blanks were arranged in a consistent systematic pattern with 12 blanks on each plate arranged in such a way that there was at least one blank present on every row and every column.

Scientific name	**Tissue condition**	**Mock community name**	**Final concentration (ng/µl)**
Cyprinidae	Fresh	Carp	20.48
Oncorhynchus mykiss	Fresh	Trout	10.24
Bolitoglossa franklini	Frozen	Salamander	10.24
Agalychnis callidryas	Frozen	Red eyed tree frog	5.12
Mus musculus	Frozen	Mouse	5.12
Lathamus discolor	Frozen	Swift parakeet	2.56
Cyanistes caeruleus	Frozen	Blue tit	1.28
Neovision vision	Frozen	Mink	1.28
Stronglocentrotus droebachiensis	Frozen	Green sea Urchin	0.64
Montipu 2 roja[1]	Frozen	Coral 2	0.64
Agalychnis moreletii	Frozen	Black eyed tree frog	0.32
Gryllodes sigillatus	Fresh	Black banded cricket	0.16

Teleogryllus commodus	Fresh	Black cricket	0.16
Fernanda 7803[2]	Fresh	Sponge2	0.08
Palaemon serratus	Fresh	Prawn	0.08
Homarus gammarus	Frozen	Lobster	0.04
Fernanda 7987[2]	Frozen	Sponge3	0.02
Bombus terrestris	Frozen	Bumble bee	0.02
Diana Curaco[1]	Frozen	Coral1	0.01
Sarah LKA7[3]	Frozen	Sponge1	0.01

Table 2.1. The composition of the mock community used for this study. [1]Species not yet taxonomically assigned; sample from Colombian Caribbean and tissue provided by Diana Ballasteros. [2]Species not taxonomically assigned at time of donation and provided by Ferdanda Azevedo. [3]Species not taxonomically assigned at time of donation and provided by Sarah Griffiths.

Table 2.2. Experimental markers used in this study and the blocking primers used.

Marker name	Target taxa	Forward sequence	Reverse sequence	Reference
12sV5	Vertebrates	TTAGATACCCCACTATGC	TAGAACAGGCTCCTCTAG	(M De Barba *et al.*, 2014)
16sMAV	Invertebrates	CCAACATCGAGGTCRYAA	ARTTACYNTAGGGATAACAG	(M De Barba *et al.*, 2014)
HomoB	Human blocking	CTATGCTTAGCCCTAAACCTCAACAGTTAAATCAACAAAACTGCT-C3	NA	(M De Barba *et al.*, 2014)
MamMAVB1	Mammals	CCTAGGGATAACAGCGCAATCCTATT-C3	NA	(M De Barba *et al.*, 2014)
Euka-02	Eukaryotes	TGGTGCATGGCCGTTCTTAGT	CATCTAAGGGCATCACAGACC	(Guardiola *et al.*, 2015)
OBS1	Lutra	CTATGCTCAGCCCTAAACATAGATAGCTTACATAACAAAACTATCTGC-C3	NA	(Kumari *et al.*, 2019)

The optimised PCR reactions were performed with a volume of 10 µl using amplitaq gold 360 2x master mix with 0.1% DMSO added. The primer concentrations were 200 nM for all markers (Table 2.2). As the positive controls were imbedded in other experiments, the cycle number was optimised for the experiments that the controls were imbedded in, although the cycling profile was the same for all experiments. The cycling profile for the Eukaryotic marker included an initial denaturation time of 10 mins at 95˚C followed by 21 cycles of 95˚C for 30 secs, annealing 55˚C for 30 seconds and 1 min extension at 72˚C, and a final extension time of 10 mins at 72˚C. The cycling profile for

the vertebrate marker was 95 ˚C followed by 40 cycles of 95 ˚C for 30 seconds, 48 ˚C for 30 seconds, and 72 ˚C for 30 seconds, followed by a final extension of 15 mins at 72 ˚C. Finally the invertebrate cycling profile was 95 ˚C followed by 45 cycles of 95 ˚C for 30 seconds, 48 ˚C degrees for 30 seconds, and 72 ˚C for 30 seconds followed by a final extension of 15 mins at 72 ˚C.

Library preparation method

There were seven experimental treatment groups; Lutra eukaryotic, Lutra vertebrate, Lutra invertebrate, Lontra eukaryotic, Lontra vertebrate, Lontra invertebrate, skink eukaryotic. Each experimental treatment group included at least 48 replicates (except one dataset that consisted of 27 replicates – skink study) of the same mock community, and each treatment group underwent the same laboratory analysis protocol, as outlined in Figure 2.1. PCRs were pooled by treatment group. The concentrations of the pools were then normalised to a target of 25 ng/µl where possible, however if the final pool was a lower concentration (one of seven pools was lower at 6.8 ng/µl) it was put through library preparation as is. The library preparation started with a bead clean up using 1 µg of DNA in 50 µl, as specified within the PCR free kit protocol (Illumina). The protocol was followed until the size selection step. The size selection step in the Illumina protocol was replaced with a double bead clean up at 1.8:1 ratio for markers above 150bp (eukaryote and vertebrate markers) and a 2:1 ratio for markers below 150bp (Invertebrate marker). The protocol was followed until the final double bead clean-up ,which was adjusted to a 1.8:1 ratio rather than the 1:1 ratio, as amplicons are often substantially smaller than the 350bp fragment the kit was designed for.

Quantification and normalization

The quality of the libraries were confirmed using three methods that determined the necessity of equipment that is often unavailable in many laboratories. The first approach, currently recommended by Illumina, consisted of using a bioanalyzer high sensitivity chip to check library quality. This was followed by qPCR with specific primers that bind to the p5 and p7 adapters to quantify the double adapted amplicons (NEB Quant kit). In

ligated libraries, amplicons with two adapters represent only a proportion of all amplicons present. Amplicons with only one adapter or no adapter are also present but cannot bind to the sequencer. The ratio of these three groups is important because if unaccounted for it leads to over estimation of the number of double adapted amplicons in the library resulting in an underloaded flow cell and low sequencing yield. The second approach replaced the qPCR kit with Qubit and quantified the library concentration with a combination of the Qubit output and the bioanalyzer trace. This is possible because the bioanalyzer trace has three distinct peaks; one peak of non-adapted amplicons, one containing amplicons with only one adaptor and one with both adaptors present. By taking the relative proportions of those peaks it is possible to adjust the library concentration to include only amplicons with two adaptors. For example, if the bioanalyzer trace showed peaks where amplicons with two adaptors accounted for 50% of all amplicons, and the Qubit quantified 20 ng/µl, then the Qubit output is twice the concentration of useful amplicons in the library. Therefore, there needs to be an adjustment to account for the other unusable 50%, making the true library concentration 10 ng/µl. The third and final approach quantified and normalised libraries without the bioanalyzer or qPCR. Instead, a 2% agarose gel was used to visualize the three groups of amplicons (no adapter, one and two adapters) and separate them into three bands on the gel. A rough ratio of the three groups was estimated by looking at the intensity of the bands and adjusting the Qubit scores accordingly.

Post sequencing analysis

The samples were bioinformatically cleaned and analysed using the OBITools package (Boyer *et al.*, 2016). First the samples were aligned using illuminapairedend function and the aligned reads were demultiplexed using ngsfilter allowing for no mismatches in the tag sequences and one mismatch in the primer sequences. After, the samples were dereplicated using obiuniq and mOTUs. The samples with fewer than 5 copies and read length shorter than the expected minimum fragment size (100bp eukaryotic, 80bp vertebrates and 30bp invertebrates) were removed from the dataset using obigrep.

The samples were then taxonomically assigned using the ecotag function. The reference database was constructed from the EMBL reference database (release March 2022)

(https://www.ebi.ac.uk/). The final databases, constructed as shown in OBITools3 (Boyer *et al.*, 2016), contained 51,417 unique species for them eukaryotic marker, 46,159 for the invertebrate marker and 20,003 sequences for the vertebrate marker. After assignment, only samples that matched at 98% or higher were kept. The resulting taxonomically assigned sequences were imported into R (version 4.0.3) and analysed with the metabaR package (Zinger *et al.,* 2021) to remove chimeras, tag jump and other forms of contamination. In the cases where closely related species could not be resolved the recounts were pooled and the species assigned to the lowest common taxonomic rank (e.g. Coral 1, 2, and 3 were assigned as Coral).

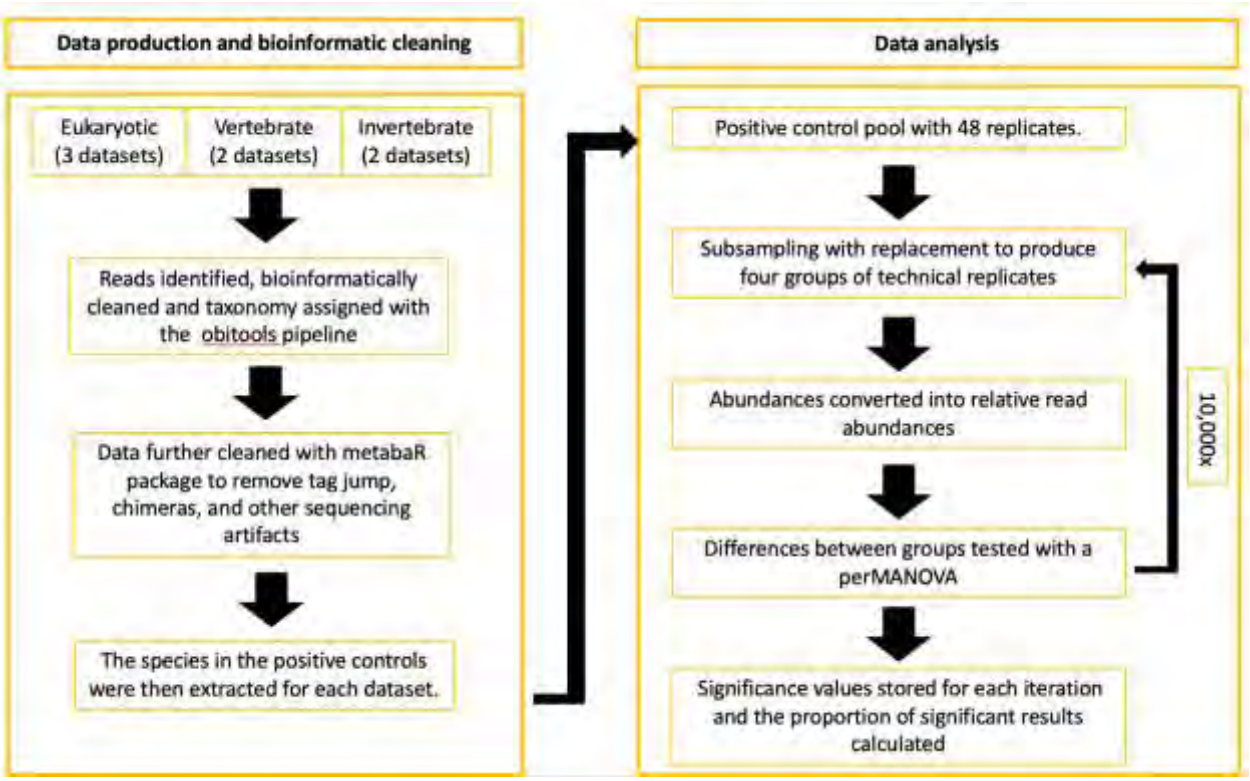

Figure 2.1: A flow diagram setting out the data production, cleaning, and analysis of the method to determine its accuracy and precision with seven different data sets that included three different markers Eukaryotic 18s, Vertebrate 12s and invertebrate 16s.

The accuracy of the method was assessed by comparing the observed read abundances for each of the datasets against the abundances of a theoretical dataset (described below).

If the method accurately recreates the abundances in the mock community, the mean relative abundance of species should be the same in both the observed and theoretical datasets. To obtain the theoretical datasets, we simulated 48 read abundances for each species to match the number of technical replicates present in the observed datasets. The theoretical read abundances were generated based on the average number of reads of the most common species in the observed mock community (carp). Values were generated in R using the rnorm function with a standard deviation set at 10% of the mean. This was repeated for each species descending at a log2 scale, so that each species expected mean was relative to the most common species.

To determine the precision of the method, the databases from the seven treatment groups were analysed from three different sequencing runs on two different platforms (NextSeq: Lontra vertebrate, Lontra invetebrate, Lutra vertebrate, Lutra invertebrate; MiSeq 1 skink; MiSeq Lutra eukaryotic, Lontra eukaryotic). For each marker in each sequencing run, we extracted the species that were known to be present in the mock community, thereby excluding any cross contamination from other samples in the run.

In order to determine the importance of number of technical replicates, we randomly subsampled (with replacement) 2, 3, 4 or 5 technical replicates (including numbers of reads for each species in the mock community). This was repeated four times creating four groups to compare how precision was affected by the number of technical replicates. At this stage the read counts were converted to relative rank abundances within each technical replicate using the decostand function from the vegan package. This process was repeated 10,000 times for each number of technical replicates (2, 3, 4 or 5) and carried out for the seven datasets. The randomly assigned communities were tested against each other using a perMANOVA, and the proportion of significant results verses insignificant was calculated from the 10,000 p values of each number of technical replicates. Any significant result would indicate the method was introducing false diversity into the samples as all technical replicate groups should all be the same. The proportions of significant results for each number of technical replicates were then recorded to show the effect of an increasing number of technical replicates for reproducibility in our method.

Finally, to determine if the obtained datasets accurately reconstructed the theoretical communities, we tested the seven datasets against the simulated data using a similar method outlined above to test precision. For each obtained dataset, we subsampled two sets of three replicates and at the same time the theoretical community of the same marker was subsampled in the same fashion. These four groups were then tested for differences using a perMANOVA. This process was repeated 10,000 times for each dataset and the proportion of significant results calculated.

Results

Quality control steps

Of the three runs, only the NexSeq run used all the usually recommended quality control steps. For this run we used a 2x125 mid-output flow cell, which yielded more than 100% of the expected output with >80% of bases called higher than q30, which Illumina counts as a successful run. The first MiSeq run was completed with no qPCR quantification of the final libraries and the quantification was achieved through adjusting the library concentration from the Qubit based on the ratio between the peaks shown on the bioanalyzer. This run generated 4,000,000 reads from a v2 2x 250 microflowcell with 97.8% bases >q30, which is the maximum Illumina expects from this flow cell. The second MiSeq run was also conducted without qPCR quantification but the Vertebrate marker was checked for quality only on a gel and the ratio of double adapted to single adapted products was determined by the intensity of the bands (in this case 50:50 double adapted to single or non adapted amplicons). In this run using a v2 2x250 flow cell we had 96.4% of bases >q30 with 13,216,116 aligned reads meaning we reached the expected output Illumina would expect from this flow cell. We also found that the proportions of the reads for each marker were as expected with each occupying a third of the total number of reads, demonstrating there was no loss of precision when using the alternative library validation steps.

Accuracy

We were able to accurately reconstruct the community structures of the three markers across all runs, although there were some differences in abundances of some species

relative to the expected community structure (Figure 2.2). In the case of the Eukaryote marker, the two mammalian species were either not identified (mink) or disproportionately low in abundance relative to the expected abundance (mouse) (Figure 2.2A). Mammalian species were less abundant than designed showing potential bias of the Eukaryotic marker for species of that taxon. With the Eukaryotic marker, there was an issue distinguishing between the two frog species *Agalychnis callidryas* and *Agalychnis moreletii,* which is unsurprising as the species belong to the same genus and are known to hybridise at the site where our samples were collected (Preziosi, personal observation). There was a wide standard deviation in the samples (Supplementary, excel 2) , but this was due to variation in read depth in the technical replicates, with low abundance for one species being associated with low abundance for all other species

within the same technical replicate or vice versa, i.e. the correct relative abundance of species was preserved within technical replicates (Fig. S2.1, S2.3 and S2.3).

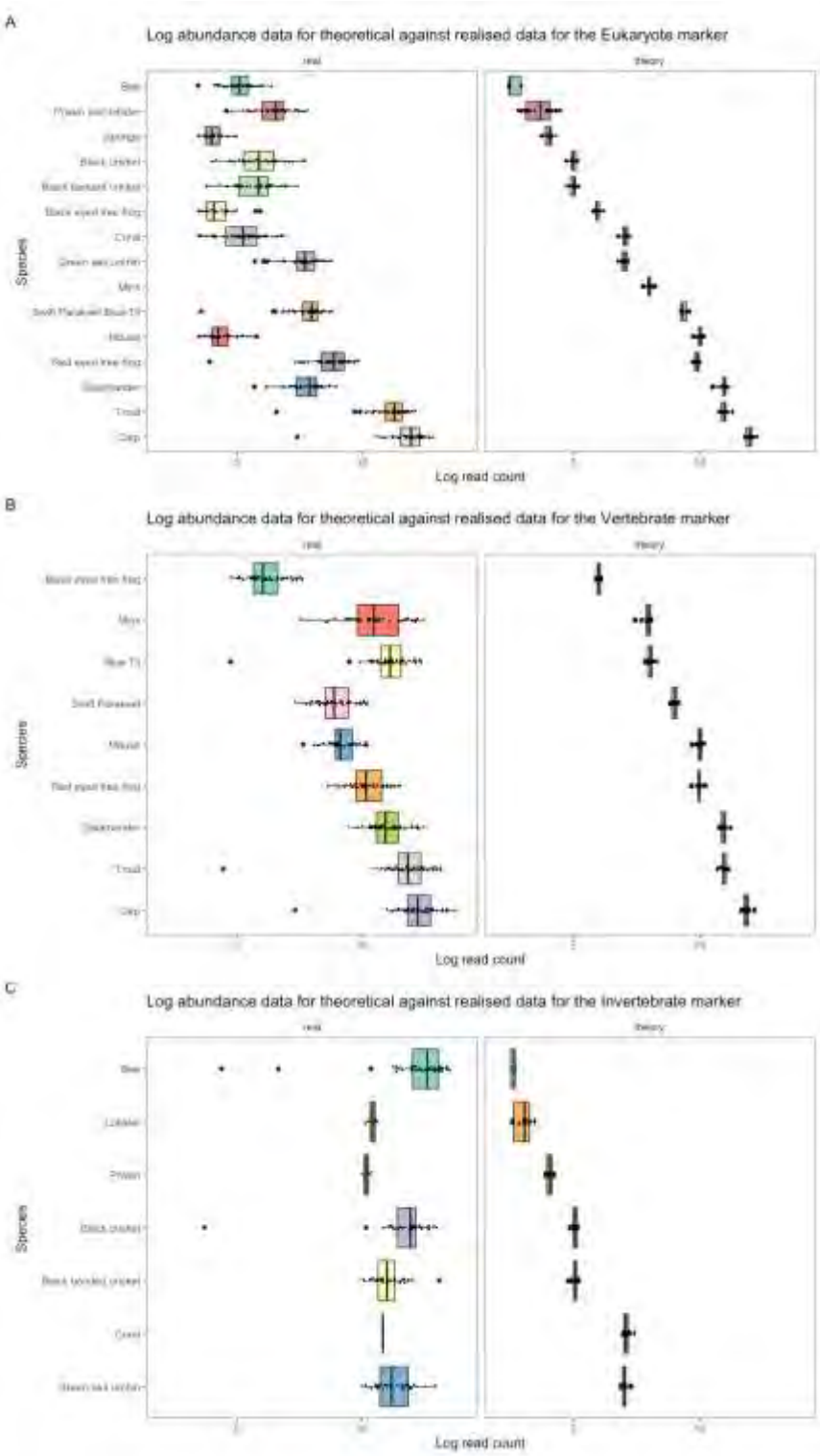

Figure 2.2 Comparison of the realised abundances for three markers using our method compared to a theoretical distribution that would have been amplified if the method was 100% accurate in reconstructing the distribution of a mock community of our manufacture. A: Eukaryotic marker. B: Vertebrate marker. C: Invertebrate marker. The X axis is on base 2 scale as the theoretical distribution was constructed on this distribution.

With the vertebrate marker, the observed distributions were accurately reconstructed with the exception of Blue Tit and Mink reads, which were present in higher abundances relative to what was expected (Figure 2.2B). All other species were present in the correct proportions and the relative abundance pattern was preserved within technical replicates. The invertebrate marker reconstructed the observed distributions (Figure 2.2C). However, several species did not follow the expected relative abundance distribution. Coral was underrepresented and only present in a few technical replicates, while bee and black cricket was overrepresented in general. The invertebrate marker dataset had less consistency within technical replicates than was present in other markers (Supplementary Figure S2.3). However, all invertebrate species in the mock community were present and the general distribution follows the theoretical distribution overall.

The observed datasets did differ significantly from the theoretical communities (Table 2.3). The subsampled observed datasets were always significantly different when compared to the theoretical communities for all seven data sets and therefore for all markers.

Table 2.3: Proportion of perMANOVA tests that returned a significant result out of 10,000 random sub-samplings of the theoretical and obtained datasets. perMANOVAS used relative rank abundances converted into Bray-Curtis dissimilarities with 1000 iterations.

Species	Marker	Percentage of significant results
Skink	Eukaryotic	100%
Lutra	Eukaryotic	100%
Lutra	Vertebrate	100%
Lutra	Invertebrate	100%
Lontra	Eukaryotic	100%
Lontra	Vertebrate	100%
Lontra	Invertebrate	100%

Precision

The level of precision is extremely high with a minimum of 95% of the perMANOVAs being insignificant for all the datasets tested with any number of technical replicates regardless of the marker or platform. The highest percentage of replicability being with two replicates, which was true when accounting for marker and sequencing effects (Figure 2.3). However, two replicates was the number of replicates with most variance and for true consistency, a higher number of replicates is required with the full convergence being achieved with five replicates. Overall, three replicates is sufficient to be sure of consistent results between technical replicates for most samples, although detection limits may become an issue with lower numbers of technical replicates (Figures S2.1, S2.2, S2.3). We found few or no reads of the species with the lowest abundances in our technical replicates indicating as expected the chances of amplification are affected by initial sample concentration. This was further underlined by the slightly lower proportion of invertebrate perMANOVAs that were insignificant. This correlates with a higher proportion of that community being at very low concentrations in the mock

community indicating that the abundances and presences of some of the species using 7the invertebrate marker were less consistent compared to the other markers.

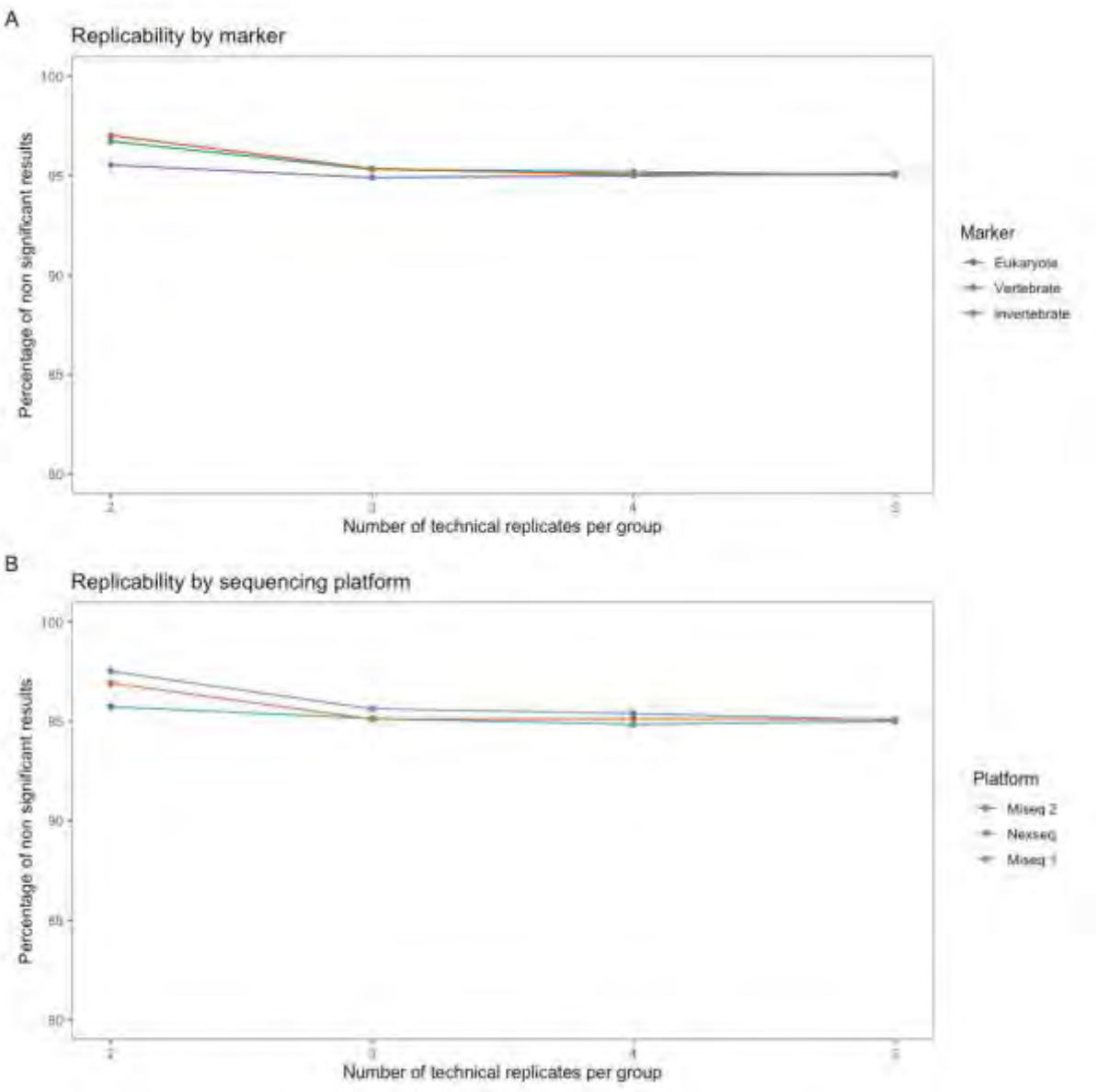

Figure 2.3: A comparison of the replicability of the method with differing numbers of technical replicates from two to five. A) marker effect and B) run effect. For each number of technical replicates there were four groups randomly subsampled with replacement from a pool of technical replicates. These groups were tested for differences using a perMANOVA with Bray Curtis distances and 1000 iterations. Pools were sampled 10000 times in total for each set of

technical replicates and the proportion of insignificant results calculated. We demonstrate a minimum of 95% replicability for all markers across all runs indicating a high level of precision using our method.

Discussion

Developing accurate and cost-effective methodologies for preparing samples for sequencing is to produce accurate and informative insights into the status of ecosystems, something that is of critical importance in a world currently undergoing a biodiversity crisis (Speaker *et al.*, 2022). Despite the decreasing cost of sequencing, the need for highly equipped laboratories and a drive to increase experimental robustness with greater numbers of controls and technical replicates has made it increasingly difficult for institutions that are less well equipped than those driving this increase in standards, and this has meant many laboratories can find it difficult to conduct metabarcoding studies (Tedersoo *et al.*, 2022). This is of real concern as often the labs that lack the necessary equipment are located in the very places that contain the highest levels of biodiversity and are currently undergoing the highest rates of biodiversity loss (Rodríguez *et al.*, 2007). Creating methodologies that can meet the current standards required by the field while remaining cost effective and that can be carried out even in the absence of specialized equipment is a high priority.

Here we present a method that is based solely off commercially available reagents, which are always sold where Illumina sequencers are available and can produce precise and accurate datasets at a highly cost-effective price. We found that our method accurately reconstructed abundances of a mock community we manufactured, and that repeatability was 95% or higher, even with low numbers of technical replicates. This pattern was consistent with three different markers on three different sequencing runs and on two different Illumina sequencing platforms. The consistency of these results gives confidence that we can reconstruct the communities from real samples with high confidence, assuming a good marker choice for the questions being asked and the target taxa. We also find in our results that as we approach the lower detection limit, as we did with some of the least abundant species in our mock communities, that both the chances of detection and the accuracy of the abundances suffer as a result. This has implications

for the detection of rare species in metabarcoding studies and we suggest that positive controls should not only include a variety of relevant taxa at differing concentrations to investigate potential bias in amplification and sequencing, but also that this concentration range should extend down to potential detection limits. Once species in the positive mock community controls become inconstantly detected it may be prudent to set that read count as a lower limit for ecological analysis, as species detected below such counts may be inconstantly detected and, depending on the question, have the potential to bias results. Such analysis could be added without significantly increasing cost or major changes in current experimental designs.

The marker structure used to investigate our method brought to light some artifacts of the metabarcoding process that may be of importance to many studies. The invertebrate marker was able to successfully amplify all species, even those at negligible concentrations were amplified in a number of the technical replicates. However, the underlying pattern of relative abundance between the species became muddled. This could be due to the low copy numbers being more susceptible to the stochastic variation of early PCR cycles, as has been discussed in previous studies (Shirazi, Meyer and Shapiro, 2021). Despite this, the overall read counts of the invertebrate marker are comparable with that of the vertebrate marker, which is a by-product of the PCR and library preparation methods themselves as they normalise all libraries to the same concentration before sequencing. This means the presence of a hierarchical marker is essential when comparisons will be made between taxa identified using different markers as the abundances will look similar between such markers even if, in the original sample, the underlying abundances were very different. This has significant implications for many studies as using multiple markers to investigate taxa is standard practice in many fields (da Silva *et al.*, 2019; de Groot *et al.*, 2021; Topstad *et al.*, 2021).

The method presented here is extremely cost effective and produces reliable data using only commercially available reagents, which are sold by Illumina wherever they sell sequencers. Furthermore, we demonstrate that it is possible to prepare these libraries consistently in the absence of the equipment recommended for the quality control stages of library preparation by presenting alternative solutions that are available at much lower cost and with less specialized equipment. For example, bioanalyzers are not sold in many regions, including Latin America, meaning libraries cannot be checked for quality in the way currently recommended by Illumina (*TruSeq DNA PCR-Free | Simple prep for*

sequencing complex genomes, 2022). Here we show it is possible to validate libraries by gel photos and Qubit alone. We also were able to quantify libraries with Qubit along with a gel or bioanalyzer trace, which potentially reduces the need to import expensive custom qPCR quantification kits in countries where there is no direct supply of such reagents. We hope that publicising such workarounds may open our methods to laboratories that previously felt unable to take on such studies due to lack of equipment.

We found our method to be precise, we did however face issues when interpreting accuracy; the general community structure was reconstructed but certain taxa were either over- or under-represented in all markers. For example, the accuracy of the Eukaryotic marker for mammalian samples was poor but it is impossible for us to determine the exact cause of this inaccuracy. There could have been issues with the mock community manufacturing process due to DNA quality issues, inaccurate quantification or pipette error, all of which are known causes of bias in genetic studies. Beyond the manufacture of the mock community there is also the effect of marker bias causing the relative abundances of the community to change, which has been well documented in other studies (Krehenwinkel *et al.*, 2017). Such inaccuracies in our study make it extremely difficult to isolate their source. Other methods of producing mock communities, such as synthesising long oligos, may have minimised manufacture bias, while it may have been possible to analyse a community without amplification to remove the effect of marker bias. However, this would have represented an unrealistic test of the method and therefore such methods were not pursued.

This method, like other ligation-based methods that have previously been published, has the advantage of being cost effective in the sense of a very low cost per sample, far lower than the most widely used method for metabarcoding using two step PCR (Bohmann *et al.*, 2021). It has been noted that methods such as ours suffer from potentially high levels of tag jump, where a sequence can jump from one sample to another through the incorrect end repair of the identifying tags present on the forward and reverse primers, with potential rates of tag jump affecting as much as 40% of the reads produced (Carøe and Bohmann, 2020). This has serious implications for the reliability of many ligation-based methods including ours. The cause of most tag jump is poor PCR optimisation and the end repair stage of library preparation and, as such, it has been recommended to

remove the end repair step using bespoke ligases (Schnell, Bohmann and Gilbert, 2015). We were unable to attain sufficient library concentration with commercial reagents to create successful libraries, as the ligation efficiency dropped dramatically in the absence of this step. Our aim was to create a protocol available to laboratories that may struggle to obtain specific ligases from suppliers at a reasonable price due to lack of availability, therefore we do not omit this step resulting in higher rates of tag jump in our samples, between 2-7% on average. We believe this to be manageable levels and the tag jump filtering methods available were effective in cleaning our samples. However, where such ligases are easily available our quality control steps can easily be combined with such methods improving overall results. We are confident that, despite the limitations, the method we have developed is cost effective and can provide robust results especially if good laboratory practice is followed.

Data availability,

All supplementary information files are available in a public repository on Zenodo.com https://doi.org/10.5281/zenodo.8060380 raw sequencing files will be available upon publication.

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Supplementary information

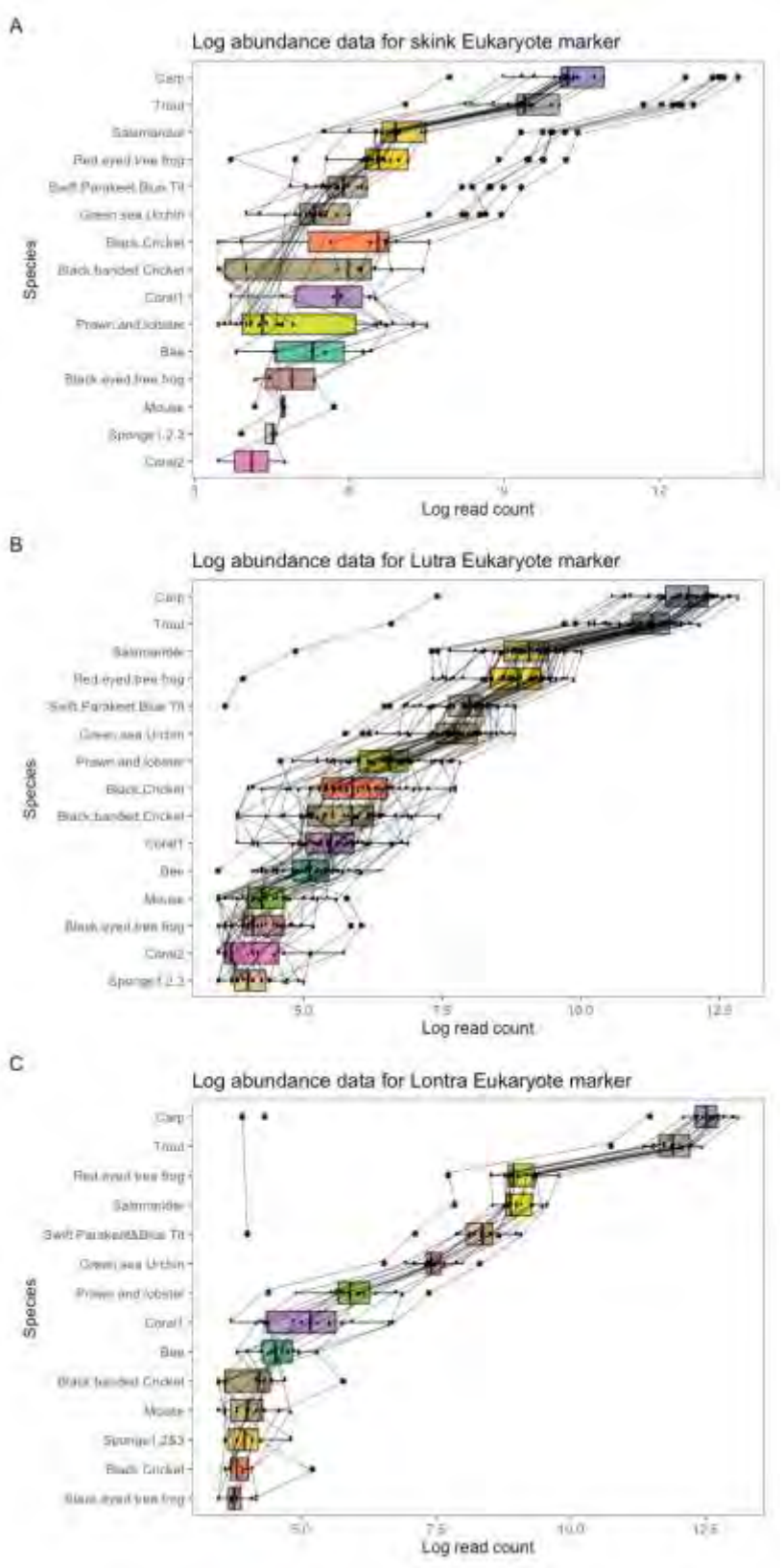


Figure S2.2.4: Comparison of the accuracy of the method with the three eukaryotic experimental treatment groups. Where the relative abundance of each of the obtained species for each of technical replicates are linked by a line this shows that the rank order is often preserved within technical replicates especially with abundant species.

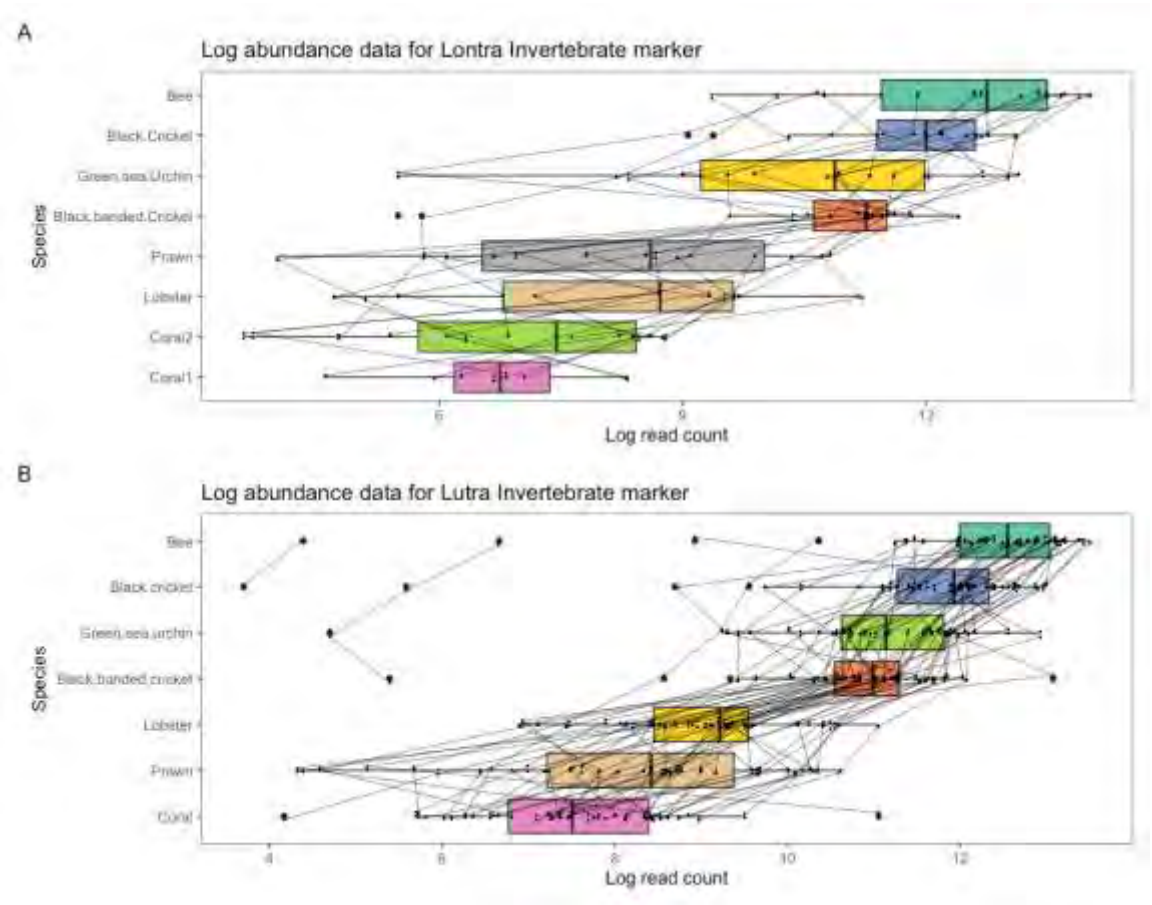


Figure S2.5.2: Comparison of the accuracy of the method with the two Invertebrate experimental treatment groups. Where the relative abundance of each of the obtained species for each of technical replicates are linked by a line this shows that the rank order is often preserved within technical replicates especially with abundant species.

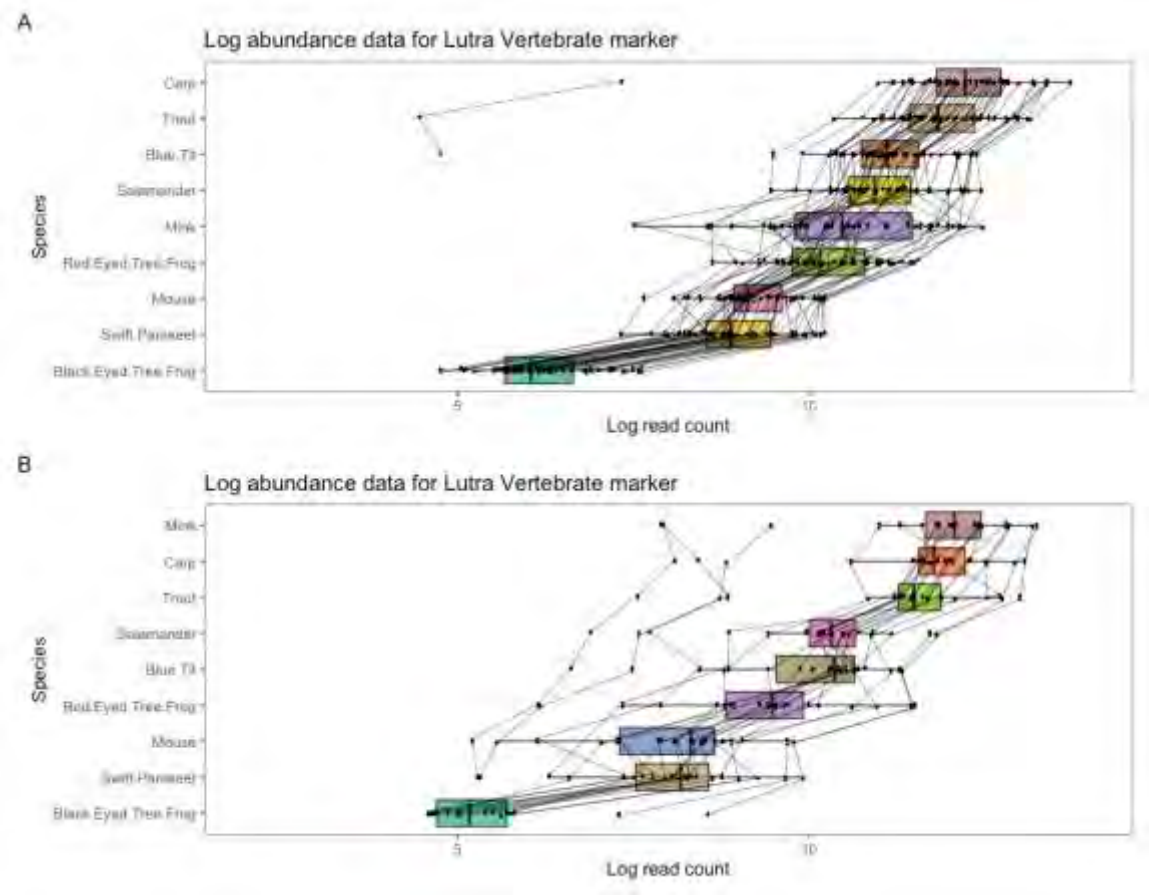


Figure S2.3: Comparison of the accuracy of the method with the two vertebrate experimental treatment groups. Where the relative abundance of each of the obtained species for each of technical replicates are linked by a line this shows that the rank order is often preserved within technical replicates especially with abundant species.

Chapter 3 A systematic review of the literature on *Lutra lutra*, the Eurasian Otter

Abstract

Identifying and filling gaps in our understanding of species and ecosystems is an essential task in order to further conservation, as good understanding makes it conservation efforts more likely to be effective (Sutherland *et al.*, 2004). However, often research is not always focused on the areas where knowledge is most lacking leading to inefficient use of research efforts. *Lutra lutra*, the Eurasian Otter, provides a good case study for this as it is a well-studied species and potentially a flagship species for conservation. Here we evaluate whether the body of research on *L. lutra* provides a comprehensive overview of the species. We identified 750 papers published on *L. lutra* between 1950 and 2021 with the only limitations being that they were peer reviewed and that *L.lutra* was the main focus of the paper. This substantial volume of research should mean that we have an excellent overview of otter ecology and status. However, the research is geographically biased to a small part of the species range and focussed on relatively small number of topics. Nine percent of all papers published on the Eurasian Otter are review articles. Overall, this results in large gaps in our knowledge, both topically e.g. otter behaviour, and geographically, with virtually all of Asia being underrepresented. These imbalances in our understanding have the potential to hamper conservation efforts. Finally, we believe this issue is not constrained only to *L. lutra* but may be part of a more general pattern in conservation research that needs further investigation.

Introduction

L. lutra has a one of the largest species ranges of any mammal in the world, stretching from Western Europe to the east coast of Russia and as far south as Sri Lanka (Yoxon and Yoxon Iosf, 2019). It is currently classified as Near Threatened (IUCN/SSC Otter Specialist Group, 2021), with recoveries in European populations being balanced by increasing threats to populations in Asia (Gomez et al., 2017). As a semi aquatic

mammal, otters provide a highly informative window on freshwater ecosystems that a huge number of species, not least humans, depend on for survival (Zacharias and Roff, 2001). The precipitous declines that *L. lutra* underwent in the 1970s due to the increasing levels of persistent organic pollutants (POPs) demonstrated how this species can be an effective indicator for freshwater ecosystem health (Smit *et al.*, 1998; Prigioni, Balestrieri and Remonti, 2007). This unique position as a widely distributed species in a key ecosystem has driven many publications investigating many aspects of otter biology, including population size and structure (Hájková *et al.*, 2009). Despite the perceived upturn in the fortunes of the Eurasian otter (Conroy and Chanin, 2000), this success has largely been confined to Western Europe with data on its status across large areas of its range being unreliable but often show declining populations (Basnet *et al.*, 2020). This further underlines the need for an evaluation of the literature to determine the most effective way to focus limited research efforts for the effective conservation of *L. lutra* (Stewart, Coles and Pullin, 2005).

The large numbers of papers published on *L. lutra* have precipitated many reviews on specific topics or over geographical portions of the range, such as diet and genetic methods (Lampa *et al.*, 2013; Reid *et al.*, 2013), and population size and distribution (Conroy, Melisch and Chanin, 1998; Prigioni, Balestrieri and Remonti, 2007; Hájková *et al.*, 2009). These reviews have added valuable insight on the aspects of otter biology, standardising methods and providing standard collection protocols, for example (Parry *et al.*, 2013). Furthermore, there have been several reviews synthesising the information we currently possess on *L. lutra* with the aim to provide a full account of what we know about this species (Yoxon and Yoxon Iosf, 2019). However, none of these reviews have systematically categorised the literature to identify the topical and geographical patterns that have developed overtime and thus identify knowledge gaps. Here, we focus on the general trends in the literature itself to identify areas that have been well studied and those that have not, with the aim of providing priorities for future research (Stewart, Coles and Pullin, 2005).

All peer reviewed papers published on *L. lutra* from several databases (Google Scholar, Web of Science, and The IUCN Otter Specialist Group) we collected, following PRISMA guidelines (Page *et al.*, 2021). They were then categorised them by broad topic, specific focus of the study, data collected in the study, methodologies used in the study, and location of study, in order to determine patterns in research topics as well as

determining the types of sampling most undertaken. Our initial searches identified over 1319 papers, and of these 750 were taken forward into our study with the rationale for inclusion or exclusion spelled being whether they were peer reviewed and if the full manuscript was accessible. Only papers pubished in English were despite searching for studies published in other languages as those couldn't reliably evaluated . These papers were analysed, and we have identified several topics that have been well covered as well as some that have been relatively understudied, such as behaviour. As a result, we have identify several areas in need of further study while highlighting subjects that may benefit from reassessment with more modern methodologies.

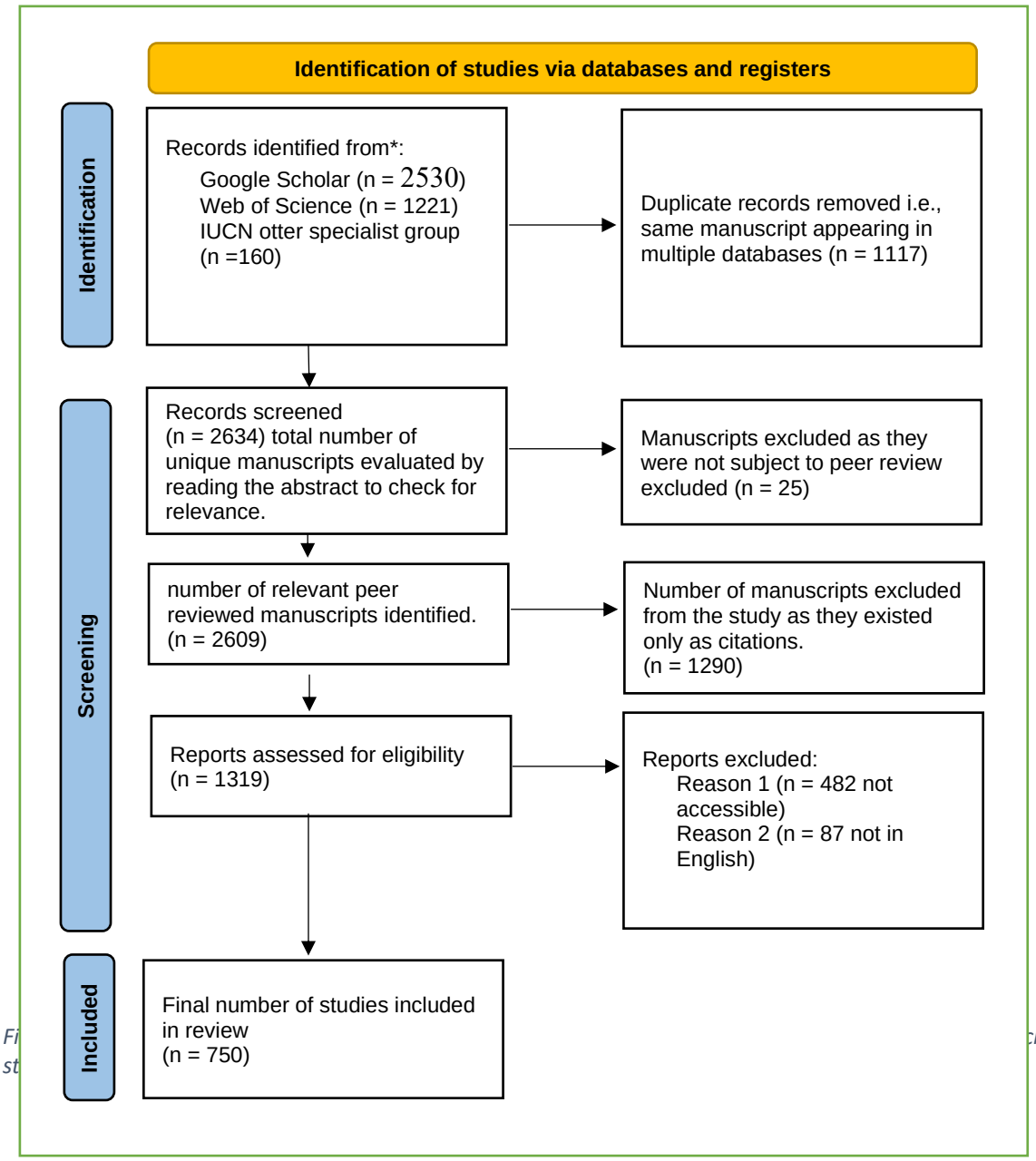
Here the following questions are investigated:

(1) What topics have been investigated most on *Lutra lutra* and has the research focus changed over time to reflect current threats to the species?

(2) Does the peer reviewed literature effectively cover all major parts of the species range?

We searched the following databases for literature, Google Scholar, Web of Science and The IUCN Otter Specialist Group, using the search term "Lutra lutra" OR "European otter" OR "Eurasian otter", and confining the search to literature published between 1950-2020, inclusive. The final search of the databases was made in December 2020. The Google scholar search identified 2530 papers in total, while Web of science yielded 1221 papers. Only 104 papers identified by Web of science were not present in the google scholar results, while Google scholar identified 1117 papers not present on Web of science. All IUCN otter specialist group papers were identified by google scholar. Manuscripts that appeared in multiple databases were identified and duplicates removed, ensuring the number of papers was not artificially inflated. In total 2634 papers were taken forward from the first stage and further screened (2530 from google scholar + 104 extra papers from Web of science). The remaining papers were then checked to ensure they had undergone peer review (25 removed at this stage) abstracts of the remaining papers were then read to ensure their main topic was *L. lutra.* Papers that could not be accessed as they were only citations identified by Google scholar were removed (1290 papers) leaving 1319 papers to be assessed. Of the remaining 1319 papers 482 were discounted as the full text could not be accessed while 87 were removed as they were not

published in English and couldn't be assessed effectively leaving a total of 750 papers were taken forward for data analysis (Figure1).



Each paper was reviewed and the general focus of the article was categorised into one or more of the following pre-defined broad topics: Ecology, Behaviour, Population, Reviews or Methods, Contaminants/Pollution, Health, and Anatomy and Physiology. Definitions of broad topics are provided in Table 3.1. Papers were categorised into all broad topics they covered, so articles could appear in more than one topic, although only nine papers did occur in more than one broad category. The specific focus within each of the broad topics was also noted as a 'specific topic' (Table 3.2). For example, within ecology a study could have focused on diet or habitat use, and this information was recorded in the specific focus category. As was the case for broad topic, if the paper met the criteria for two specific focuses it was included in both subcategories. All criteria were decided before information was collected. The type of data collected in each study was recorded e.g., tissue or faecal samples. Finally, where given in the paper, the country or countries and number of river basins the study collected data from were recorded. In the UK, more localised information of county where data were collected was also recorded. All metadata collected are provided in Supplementary Data 3.1.

Analysis of the data

The collected data were analysed using descriptive statistics using R studio Version 1.25033, and the packages tidyverse (Wickham *et al.*, 2019) and ggplot2 (Villanueva and Chen, 2019). Colour scales for plots were chosen from the Rcolourbrewer (Neuwirth and Gravit, 2011)package and themes created from the ggthemes package (Arnold and Arnold, 2015). The number of papers covering each broad topic and the specific topics within that topic were summed to produce summary tables of each with the number of papers that fell into each category. This table was then used to produce a bar plot of the most common broad topics published on *L. lutra* The number of papers published on each broad topic in each decade was also summarised by categorising all papers published by decade and by topic this summary table was then used to create a faceted bar plot of broad topic by decade. Temporal patterns on the topics published were investigated by grouping papers by broad topic and decade. These frequency tables were then used to produce faceted bar charts using ggplot2 (Villanueva and Chen, 2019). The temporal patterns were visualised using choropleth maps the species range was taken from the IUCN database and the number of publications per country was then calculated

and plotted on a world map using sf (Pebesma, 2018), maptools (Lewin-Koh, 2010)and rgdal (Keitt, Bivand and Bivand, 2010). The UK was considered in more detail with county level data being taken and plotted on a map from the Ordnance Survey and the number of publications per county was tallied and plotted overall and by decade. Faceted plots were produced using the patchwork package.

Category	**Broad subject definition.**
Ecology	Articles relating to ecosystem interactions between *Lutra lutra* and their abiotic and biotic environment. This includes studies on diet, habitat use, suitability and how human activity impacts this species
Population	Articles focusing on population size, history, and diversity within this species including both molecular techniques to look at genetic diversity and survey focusing on population sizes.
Reviews and Methods	Review articles that consolidate literature on a specific topic or location such as a region or state. this section also includes methodological papers describing new or best practices for performing studies of various kinds on *Lutra lutra.*
Contaminants/Pollution	Papers studying the impact of toxic chemicals or elements released into the environment and their impacts on the otter population such as the levels of these compounds or metals found in tissue or faeces.
Health	Papers published focusing on the health issues of otters including papers on diseases parasites, heart conditions and microbiomes.
Anatomy and Physiology	Papers focusing on the bodily structure of *Lutra lutra* and physiological processes such as the production chemical signals and energetic requirements.

Behaviour	Studies focusing on the actions and responses of *Lutra lutra* caused by changes in their environment for example papers on daily routine, predation behaviours, changes in habits due to human activities.

Table 3.1: The definitions of each of the subcategories within the broad subject

Specific focus	Definition
Acidification	Papers looking at the impacts of acidification on *Lutra lutra*
Camera traps	Papers on camara traps for studying *Lutra lutra*
Methods	Methods papers outlining how to perform techniques for studying *Lutra lutra*
Chemical communication	Papers looking at the chemical signalling used by otters for inter and intra species interactions
Reviews	Papers reviewing a topic covered in the literature of *Lutra lutra e.g.,* genetics or survey techniques but also including species reviews.
Daily routine	The daily habits of *Lutra lutra*
Determination of age	Papers looking at otter ages normally through post-mortems
Development	Papers looking at the development of *Lutra lutra* from juveniles to adulthood.
Diet	Diet studies looking at the prey species of *Lutra lutra*
Disease	Studies looking at disease in *Lutra lutra*
Health	Investigations looking at the general health of otters, with topics such as body condition cause of death.
Genetics	Studys looking at the genetics of *Lutra lutra* for example genomic diversity or historic population bottlenecks
Habitat use	Papers looking at the use of different habitats within the terretories of *Lutra lutra* generally looking to determine what otters need to sustain a population.
Habitat subtility	Papers which classify the quality of habitat for *Lutra lutra* often using modelling from data obtained with surveys and habitat usage papers.
Heavy metals	Papers looking at the levels of common metal contaminants such as lead, Iron, Zinc, etc
POPs	Papers looking at persistent organic pollutants such as DDT and organochlorides
Human impacts	Papers focusing on land use changes in the environment such as deforestation or urbanisation on the effects on otters.
Microbiome	Papers on the microbiome of *Lutra lutra*

Mortality	Papers which through postmortem have determined the causes of death in *Lutra lutra*
Niche occupation	Papers on the niche occupation of *Lutra lutra* through its interactions with other species often with a focus on diet
Parasites	Papers focusing on parasites
Physiology	Physiology papers looking at for example metabolism
Population distribution	Investigations into the distribution of *Lutra lutra* in different areas of the species range.
Population genetics	Papers with a specific focus on the population genetics of *Lutra lutra* normally with microsatellites
Population size	Papers which focus on the population size in each area often using surveys to estimate numbers.
Population structure	Papers using genetics to determine are their genetic barriers or if there is cryptic diversity within known populations bit that a small and large scale.
Predation	Behavioural studies looking at hunting techniques exhibited by otters.
Reintroduction	Studies looking at reintroduction programmes and documenting their success and failures
Reproduction	Papers looking at the reproductive constraints and requirements of *Lutra lutra*
Interactions	Behavioural studies looking at how individual associate with each other.
Spraining behaviour	Behavioural studies looking at where and how often *Lutra lutra deposits spraint and what factors effect this behaviour.*
Surveys	Papers reporting the results of population surveys.
Taxonomy	Papers on the taxonomic status of *Lutra lutra*
Territoriality	Behavioural papers on how territorial *Lutra lutra* is and how the species marks territory.

Table 3.2: The definitions of each of the subcategories within the broad topic, i.e. specific topic.

Results and Discussion

We collected a total of 750 papers. The number of papers has increased over time, as with published literature in general, with only 20 papers (2%) were published between 1950 and 1979, compared to 25 papers in the one year of 2020 (Table 3.3). The most studied category was Ecology with 254 papers and the second most studied area was Population (Figure 3.2). The remaining topics, Health, Reviews and Methods, Contaminants/Pollution, Anatomy and Physiology, and Behaviour, each accounted for approximately 10% of the total number of papers published.

The rank order of broad topics has mostly remained stable overtime, with Ecology being the preeminent topic in every decade from 1970 to 2020 (Figure 3; 47% papers of in 1970-1979). Population studies have been the second most studied topic, except in the 1990s when it was overtaken by research on Contaminants. In total there have been 172 papers looking at the population status of *L. lutra*, 78 of which have focused on population size estimation; other common specific topics included population genetics (31 papers) and population distribution within a geographical area. There were 84 papers in the Reviews and Methods topic, with 23 of those papers being reports on the overall status of the species. The remainder of papers in the Reviews and Methods topic were specific topics such as survey methodologies or DNA extraction techniques. The majority of species reviews have focussed on localised areas of *L. lutra's* range although several have written about the species more broadly. The fourth most researched broad topic we identified was health (78 papers), which included papers on the condition of otters such as weight, disease, and cause of death. The remaining broad topics, Contamination, Behaviour and Anatomy and Physiology, all accounted for a relatively small number of papers, less than 70 each, highlighting the imbalance in literature.

Table 3.3: Number of published papers by decade

Years	Number of papers
1950-1959	1
1960-1969	4
1970-1979	15
1980-1989	66
1990-1999	162
2000-2009	209
2010-2019	268
2020	25

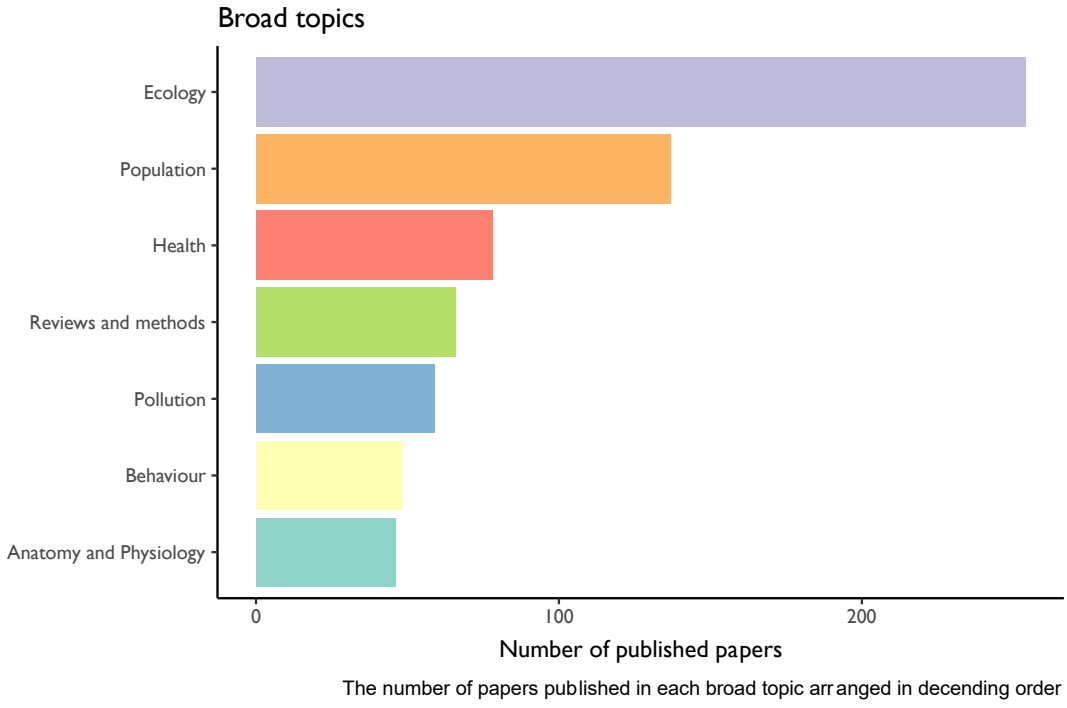


Figure 3.2: The number of papers published in each broad topic with Lutra lutra, the Eurasian Otter, between 1950 and 2020 inclusive. The total number of papers was 750.

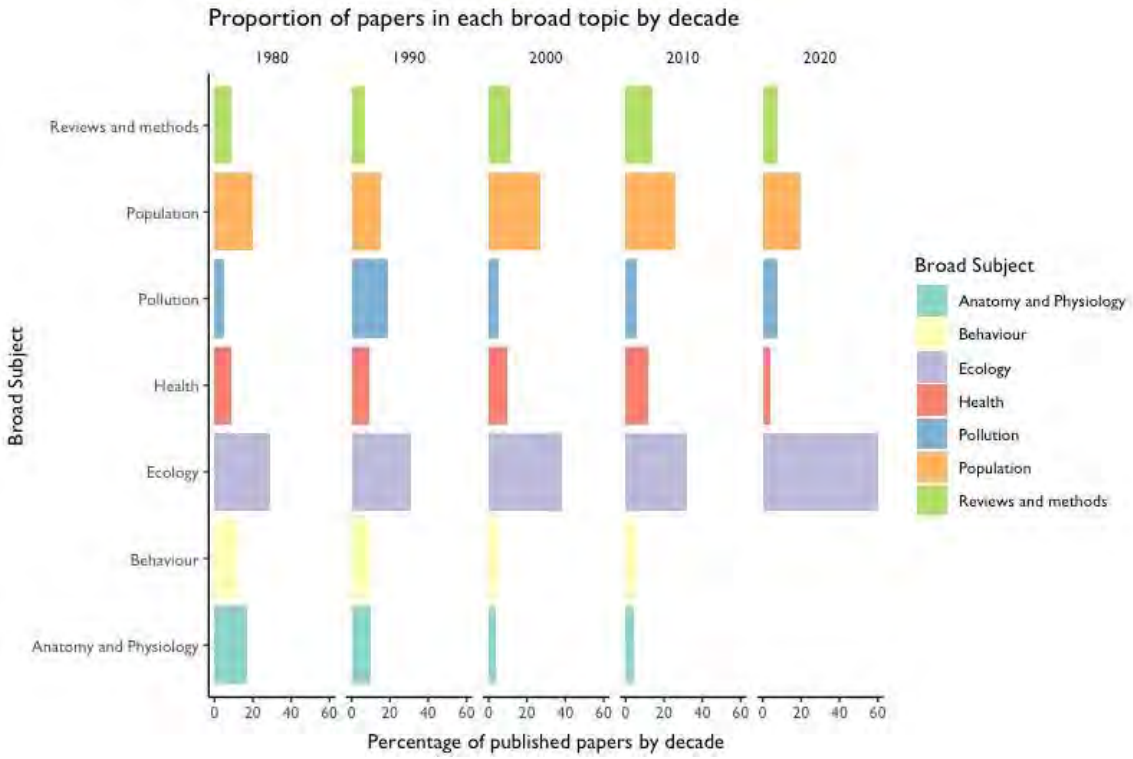


Figure 3.3: The proportion of papers published on the seven broad topics identified in this study broken down over each decade. Note papers only 20 papers were published prior to 1980 and so these are not shown on the figure.

Ecology research

Although the threats facing otters have changed substantially in the last 50 years, ecological impacts especially those induced by humans remain the largest threat that otters face (Marcelli and Fusillo, 2009; Clavero *et al.*, 2010). Accordingly, papers focusing on ecology were the most abundant in the literature with 254 in total. A substantial proportion of the ecology papers focused on diet (149 publications), so overall, we have a good general idea of otter diet, especially in the UK, where 35 papers have been published on *L. lutra's* diet. All but three of the 149 papers used traditional morphological analysis to determine prey remains. While these studies have revealed much about the diet of *L. lutra*, they are however susceptible to identification errors and

are unable to determine the relative importance of prey species (Carss and Elston, 1996; Carss and Parkinson, 1996). These studies have also tended to be local in scale, with 55% of diet studies being based on only one river or location and only 17% at a regional or national scale. This localised nature of diet studies reflects the effort required to collect and analyse the samples and would not be an issue if methods were standardised allowing easy comparison between studies, but this is not the case (Reid *et al.*, 2013). Therefore, despite the widely recognised importance of understanding diet, we currently do not have as clear a picture of *Lutra lutra*'s diet across its range. DNA metabarcoding is a new and attractive approach for increasing our knowledge of diet, as the high throughput nature of the methodology allows for large scale projects to be undertaken and it is possible to create standardised methodological protocols to allow for easier cross-study comparison than has been possible up to now (Mason and Macdonald, 1987). Several DNA based metabarcoding papers have already been published looking at diet both taking their data from Europe (Harper *et al.*, 2020; Pertoldi *et al.*, 2021) and from Asia (Kumari *et al.*, 2019). These studies were also local in scale, sampling only one river catchment. They also used different genes to evaluate the diet and generally differed in their methods, making comparisons difficult in the future. Although DNA metabarcoding gives the opportunity to address the geographic imbalance in our understanding of diet, it would be good to initially use well studied populations, such as the UK, to develop methodologies as it is easier to determine if the obtained results are line with our current understanding.

After diet, the ecological papers have focused mainly on habitat use and change; 42 papers investigated habitat use, human impacts on habitats (28 papers) (e.g. Clavero *et al.*, 2010) and papers predicting habitat suitability for otters (18 papers) (e.g. Marcelli *et al.*, 2012). Habitat use papers have mostly used data collated from population surveys where signs of otter presence is noted by surveyors in conjunction with other environmental variables, such as tree cover and land usage, to predict the suitability of areas for otter populations (Marcelli and Fusillo, 2009; Carranza *et al.*, 2012). Some surveys have used tracking either with radio-based tags or in some cases geolocation collars to investigate habitat use (Quaglietta *et al.*, 2015). Human impact focused papers have looked at the impact of conservation-based interventions, such as habitat restoration, on the otter populations using the same methods as habitat use papers (Cirelli and Sánchez-Cordero, 2009). Only two papers have been published on the effects of climate change on otter distributions and population levels, despite the huge impact

climate change may have in the future (Cianfrani *et al.*, 2011). In general, we found that the ecological literature is heavily focused on diet and further research on human impacts, especially in less well studied parts of the range, would be beneficial. Climate change may be a significant factor in otter population trends over the next century and more investigations on climate change could be key in guiding conservation actions. Where future research on diet is considered, standardised methodologies should be agreed upon to ensure cross comparison between studies is possible in the future.

Population

Determining the population size and distribution of any species is perhaps the most important information needed when acting to conserving a species. Unsurprisingly studies focusing on this topic were the second most numerous of any of the broad categories, with 172 papers in total. Population size was the most abundant subcategory with 78 papers. These were almost exclusively the result of population surveys looking for signs of otter activity, such as spraint and holts. There was a wide variety of methodologies used, despite suggested survey criteria set by the IUCN, with papers using bespoke survey methods based on their individual location and circumstances. This makes comparisons between survey locations or surveyors difficult, if not impossible to compare. Surveys were generally on a national or regional scale, unlike diet studies, but as is the case with diet studies, the variable methodologies make direct comparisons difficult (O'Sullivan, 1993). Population genetics papers started to appear in the 1990s and have become the most rapidly expanding subcategory; currently, it is the second most abundant topic with 42 papers. There have been several national studies and populations in Western Europe has been studied extensively, with population genetics being the most abundant topic published in Germany (Lampa *et al.*, 2015; Bayerl *et al.*, 2018). Almost all population genetics papers have been on microsatellites published using capillary electrophoresis and the field is still dependent on a relatively small number of microsatellite loci developed in the early 1990s, although some have been developed more recently. One paper on SNPs has been published in Germany (Bayerl *et al.*, 2018) and further investigation with this technique would be preferable to

microsatellite based methods, which show low diversity throughout the species range (Mucci *et al.*, 2010).

Many genetics papers on *L. lutra* have also looked at population structure (27 papers). These papers have more often looked at mitochondrial sequences as opposed to short tandem repeats most utilized in population size papers (Honnen *et al.*, 2015). Often such studies have looked into the evolutionary history of *L. lutra* and the demographic changes the species has undergone recently as well as the distant past (Stanton *et al.*, 2014). As is the case for population studies using microsatellites, capillary electrophoresis has been most used and other technologies have not been fully explored.

Reintroduction programmes for *L. lutra* were popular in the 1980s and 1990s when populations of the species were in severe decline in Western Europe. Six papers on reintroductions have been published, although the outcomes of these interventions in the long term remain unclear (Arrendal *et al.*, 2004). Reintroduction programmes have not been as prevalent more recently as the current outlook for the otter is not as negative as it was in that period, especially in Europe where all the reintroductions took place.

In general, population studies have focused on field surveys providing important information on the distribution and general size of subpopulations across the range. However, the variable methods used by population surveyors means that synthesis of the data are not possible. Therefore, further standardisation of studies is necessary (Kruuk *et al.*, 1986; Balestrieri, Remonti and Prigioni, 2011). Population genetic studies have found low genetic diversity, meaning future studies could focus on newer genome-wide sequencing technologies such as RAD sequencing and nanopore-based sequencers to look for diversity in different areas of the genome, although this will require the use of different sample types, as faecal samples are not usable with these technologies. The relatively small number of markers has meant that the majority of population genetics can be directly compared, making projects on a continental scale possible, such as was the case with the Europe-wide study (Mucci *et al.*, 2010). It would be a positive step if methodologies can be established with new techniques to ensure that it is possible to compare future studies with the new sequencing techniques in a similar way.

Health

Papers on the health of otters were common in the literature (11%). This is partly due to the excellent roadkill surveys that have been set up in several countries, notably in the UK, allowing large national datasets to be produced on the health of populations through post-mortem analysis (Chadwick *et al.*, 2013; Smallbone *et al.*, 2017). The most published subcategory within health was parasites, with 42 out of 78 papers focusing on this aspect. Helminths were the most common parasite found across these studies; other parasites included biliary parasites, nematodes, and ticks, amongst others (Weber, 1991; Rolbiecki and Izdebska, 2014; Santoro *et al.*, 2017). Disease accounted for 20 papers and covered a wide variety of conditions, including cancer, survival post-surgery and gut microbiome. Aside from disease, other specific topics included wounds, weight and size. Almost all these studies were from specimens which had undergone post-mortem examination (Simpson, 1997; Weber and Mecklenburg, 2000). Finally, there were five papers on mortality and three on microbiome studies. Studies on disease may be crucial in the future if an emerging virus or bacteria threatens otter populations. Equally, the monitoring of mammals for disease remains important as zoonoses and agriculturally important diseases, such as tuberculosis, are always a high priority and consistent monitoring is an important tool.

Contamination research

Over the study period, 62 papers have focused on contamination and pollution. Publications on this broad topic peaked in the 1990s, with 30 articles published in that decade (48%), driven by the precipitous declines of otter populations across Western Europe due to high levels of DDT and PCBs (Smit *et al.*, 1998; Roos *et al.*, 2001; Christensen, Heggberget and Gutleb, 2010). The most common form of contamination studied has been organochlorides, which have made up at least 50% of the contamination studies in every decade and peaked at 87% in the 1990s. PCBs have been the most common focus in organochlorides along with DDT, especially in papers published before 1990. More recently there has been a shift in focus to look at pesticide contamination, which has steadily increased in number from 2000, reflecting the lowering of PCB levels and the concurrent increasing of pesticide concentrations in the environment (Gibbons,

Morrissey and Mineau, 2015). The second most commonly researched contaminant group was heavy metals, which have been investigated in 16 papers, with mercury being the metal of most interest (Mazet, Keck and Berny, 2005). More recently some newer contaminants have been investigated, including per- and polyfluoroalkyl substances (PFAS), specifically water repellents (Roos *et al.*, 2013). There has also been a paper on microplastics which will likely become an increasing problem for otters throughout its range.

Reviews and methods

Reviews and methods made up a total of 12% (84 papers) of all papers on *L. lutra*, and 65 of these were reviews papers, meaning that 9% of all papers published on the Eurasian Otter are review articles. There have been 28 national or regional species reviews published, which looked at the current and historical status of *L. lutra* in a area, but only one global review. In the last 20 years, a small number of studies (six papers) have reviewed the genetic methods for looking at population size and diversity (e.g. Bonesi, Hale and Macdonald, 2013; Lampa *et al.*, 2013). Ecology papers made up 50% of all papers but ecological reviews were proportionally less common (25% of reviews). There have been two meta-analysis papers on diet and these were focused on the accuracy of diet studies and made more general conclusions about *Lutra lutra's* diet than the individual localised studies. However, the lack of standardisation within the primary literature made it necessary to exclude a large number of papers from these two diet meta-analyses.

Behaviour

Behaviour has been largely overlooked in the literature with only 49 of 750 papers focusing on this aspect of otter biology. This is likely due to the difficulty of observing such an elusive species. Thirty-three percent of all behaviour studies have been undertaken on the same two populations in Scotland (River Tay and Shetland), likely due to relative ease with which these populations can be observed. Of the papers that studied behaviour, the daily routine of otters was the most common topic covered (42% of

behaviour papers). There were eight behavioural studies of communication, of which seven were scent-based. Six papers investigated sprainting behaviour looking at how otters chose sprainting sites and how often those sites are used. Finally, two territoriality and two reproductive behaviour papers had also been published. Overall, behaviour has been studied very little and even daily routine, which is the most studied behavioural topic, had only observed a small number of individuals in a small number of locations, meaning there is great uncertainty about *L. lutra's* daily habits in many regions. The methods employed to investigate daily routine using trackers is very expensive and invasive, which may make using these methods widely and with a significant number of individuals unrealistic. However, new technologies, such as camara traps, are far cheaper and represent a valuable way to further our knowledge of *L. lutra's* behaviour, not just regarding daily routine other aspects of behaviour, for example communication as audio is often captured along with images. More generally, more studies on all aspects of *L. lutra's* behaviour should be encouraged especially in less well known regions, such as Asia.

Geographic Bias

There was a substantial skew in the geographic distribution of studies, with most published data being from north and western areas of Europe (Figure 4). The top ten countries in terms of publications account for only 7% of the species range as defined by the IUCN but 67% of the total publications. There has been 163 papers published about *Lutra lutra* in the UK, more than double the next country, Spain (63 papers). Only six papers have considered otters in Russia, despite it having by far the largest otter population and it accounting for 43% of the range, while no papers have been published in China despite *L. lutra* having the second highest proportion of the range 14%. We made some attempts to search literature published in Russian and Chinese languages, and these rudimentary attempts returned little literature. We do recognise that there may be more literature published on populations from these areas in other languages. In general, the number of papers published in Europe far exceeded the numbers published in Asia, despite the fact far more of *Lutra lutra's* range is in Asia (*Eurasian Otter (Lutra lutra) | IUCN/SSC Otter Specialist Group*, 2021). However, the subjects covered in the few non-English articles found further demonstrated the differences in the threats that *Lutra lutra*

faces in Asia compared to Europe, e.g. there were articles about the fur trade, a threat that is virtually non-existent in Europe and consequently is not covered in any of the 750 articles covered in this review (Basnet *et al.*, 2020). Outside of Europe, most papers (30) were published about otters in South Korea. Genetics featured heavily in the papers about South Korean and while these have added valuable knowledge, it is a small and relatively isolated part of the Asian distribution, similar to the UK. More information from central Asia is vital if we are to effectively conserve the otter in these areas.

Even within the UK, the most intensively studied country overall, we found that the spatial distribution of studies was uneven, with large areas (counties) that had received almost no publications over the last 70 years (Figure 5). Furthermore, only a small number of topics (namely Health and Population) have been covered in most counties. This is due these topics being the focus of the roadkill collection studies organised jointly between the Environment Agency and Cardiff University. These studies have provided a unique insight into the otter population of the UK but many of these studies have been offshoots of the same data set meaning they are not totally independent from each other; therefore, some counties have only really been part of one study in over 40 years.

Spatial distribution of Lutra lutra papers published worldwide

Fig. A:

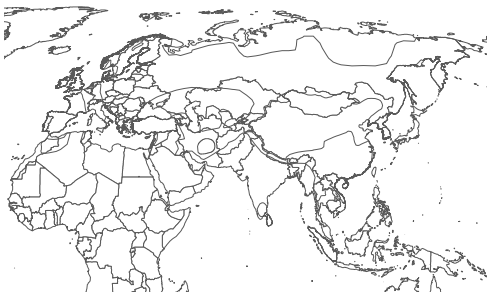
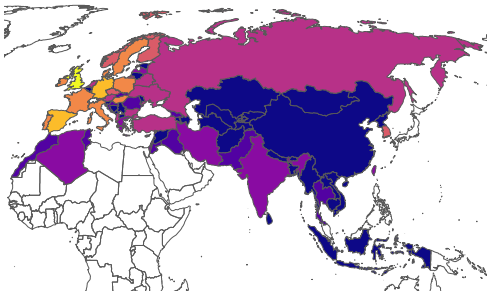


Fig. B:



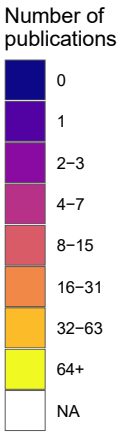


Fig. C:

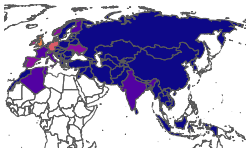


Fig. D:

Fig. E:

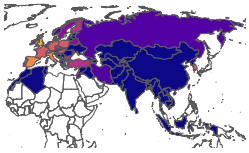


Fig. F:

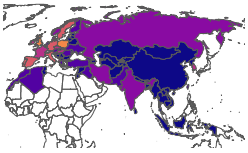


Figure 3.4: A) Range of Lutra lutra, the Eurasian Otter, based on IUCN. B) Total number of papers published in each country between 1950 and 2020. C) number of the papers published in each country before 1980. D) number of papers published in each country between 1980 and 1990. E) number of papers published in each country between 1990 and 2000. F) number of papers published between 2000 and 2010.

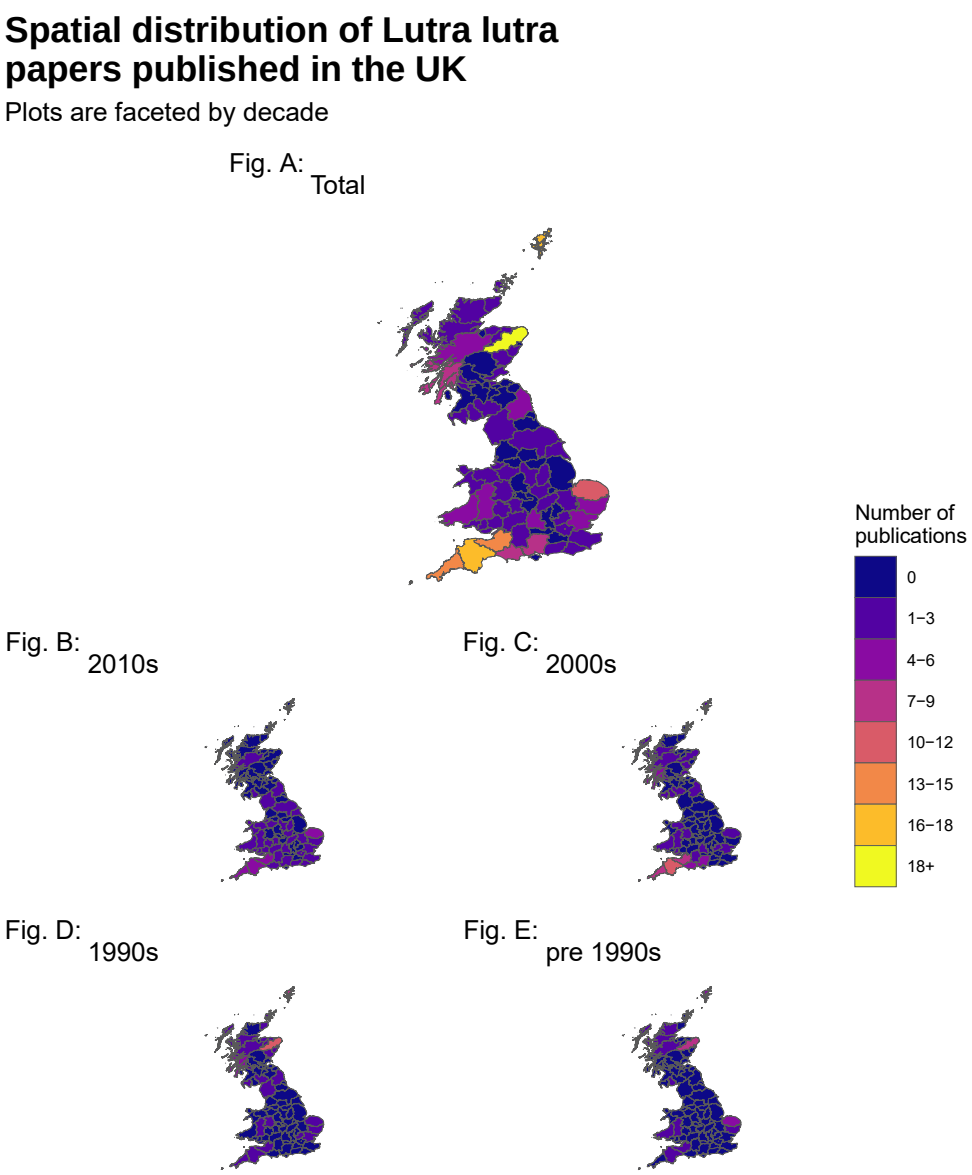


Figure 3.5: The number of papers that took data from each county by decade some counties like Cornwall and Aberdeenshire are over represented while many counties have been studied relatively rarely. A) The total number of papers published between 1960 and 2020 on Lutra lutra, the Eurasian Otter, with data collected in each county. B) number of papers published in each county between 2010 and 2020. C) number of papers published between 2000 and 2010. D) number of papers published from 1990 to 2000. E) number of papers published before pre-1990.

Gaps and future research directions

Here we show that, despite a large body of literature that has focused on *L. lutra*, there are still large gaps in our knowledge of key parts of otter biology, behaviour in particular. Contamination largely wiped out European otter populations in the 1970s and 80s, yet the topic is not among the most studied. Furthermore, there have been very few papers looking at newer contaminants such as PFAS or pharmaceutical compounds, despite the widespread concern about their accumulation in the environment (Evich *et al.*, 2022). We would strongly urge some research on these topics in any part of the species range. While ecological topics have been studied extensively in Europe, especially diet, these topics remain relatively unexplored in other areas, such as in Asia. Population surveys remain a key tool in the conservation of otters in all areas, although greater standardisation of techniques would make it far easier to make comparisons between regions. Genetically *L. lutra* has been well studied but the lack of diversity revealed by the microsatellite markers currently available leaves space for more advanced methods, such as SNP analyses, to try to investigate diversity and genetic structure at local and regional scales. Finally, metabarcoding techniques offer the opportunity to characterise the diet of *L. lutra* more accurately with the potential to create genuinely cross-comparable data so that future meta-analyses looking at diet changes over time or between different habitats possible. However, this will need collaboration and coordination between researchers to ensure standardised methods something that has not been achieved in the past.

Behaviour studies remain rare and should be prioritised wherever possible. The lack of behaviour studies may have been caused by the elusiveness of *L. lutra* and the relative cost of tracking studies making them prohibitively expensive for most researchers. But the development of inexpensive and high resolution camara traps makes it plausible to study the behaviour of *L. lutra* far more effectively than previously possible. As a charismatic species, *L. lutra* is a top candidate for citizen scientist projects and there are many members of the public in the UK already generating data on otter behaviour and diet. But this will further bias data to wealthy and densely human-populated areas of the range.

We have shown in this review that despite the large body of work looking at *L. lutra*, our knowledge does not extend evenly or adequately across the entire range. This is partly understandable as many parts of the range are sparsely populated and difficult to access. However, the ecological niche the otter occupies and its threats are different across the range, and this is especially the case between north and western areas of Europe compared to Central Asia. Increasing the number of papers on all topics in Asia should be a priority; South Korea has provided invaluable insight and it shows what may be possible to achieve in other parts of the range in the near future.

Finally, we show the uneven coverage both of topic and sampling distribution across the range has left us with significant gaps in our knowledge, which could negatively affect our ability to conserve *Lutra lutra* addressing this should be a high priority and future studies should consider where these gaps are when planning work. Furthermore, we postulate that this sort of uneven coverage may well be widespread in other species, and we recommend that studies such as ours become more commonplace in order to ensure the limited resources available to conservationists are spent effectively.

Data availability,

All supplementary information files are available in a public repository on Zenodo.com https://doi.org/10.5281/zenodo.8060380 raw sequencing files will be available upon publication.

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Chapter 4 – Use of a novel marker structure to investigate the broad diet of a mesopredator, *Lutra lutra*, the Eurasian Otter, in Eastern England

Abstract

Eurasian Otter, *Lutra lutra*, populations have fluctuated across Europe in recent centuries and currently in the UK, populations are expanding after crashing in the 1970s due to contamination of waterbodies with PCB's and DDT. Here, we investigate the diet of *Lutra lutra* using a metabarcoding approach with multiple markers to assess the relative contribution of both vertebrate and invertebrate species, as the contribution of invertebrates has been overlooked in recent metabarcoding studies of otter diet. We collected a total of 286 faecal samples from four locations in Eastern England (Rivers Alde, Blyth, Waveney, and Minsmere). The Eukaryotic marker found 53% of abundance was vertebrates, once non-metazoans were removed, and 47% was invertebrate reads. We consider that 100% of the vertebrate taxa were relevant to the diet, but only 18% of the invertebrate. This means that invertebrates conservatively make up 8.3% of the diet in terms of abundance. Fish made up 72% of the diet, birds 12%, mammals 4.5% and amphibians 3%. Of the invertebrates, shrimp and crab were the most important groups, present in up to 26% of samples. Our study demonstrates that it is important to use markers that encompass the whole diet of a species to ensure important prey items are not missed. The importance of invertebrates in the diet of otter populations in South Eastern England is an important piece of information when constructing conservation strategies for *Lutra lutra* in the UK and beyond.

Introduction

Determining and monitoring the diet of a mesopredator species in the wild provides fundamentally important information for conservation, revealing trophic interactions and providing insight into the environmental conditions a species needs to survive and what changes to the ecosystem may be occurring (Ecol, Ser and Bowen, 1997). The advent of metabarcoding allows us to investigate multiple trophic levels of the ecosystem using predator faecal samples, so that studying the diet of a carnivore simultaneously provides a window on the species that it preys upon, and potentially we can infer changes in prey populations through studying predator diet (Ruppert, Kline and Rahman, 2019). The European otter (*Lutra lutra*) represents a good case study as a biomonitoring species. It is a generalist predator that opportunistically feeds on aquatic and terrestrial species, including birds, mammals, fish, amphibians, invertebrates, and crustaceans (Krawczyk *et al.*, 2016)(Reid *et al.*, 2013). Otters characteristically mark territories with their faeces, commonly known as spraint, by leaving them in latrines, which they consistently return to throughout their occupation of a territory. Thus, the otters' diets can easily be studied as spraint collections are predictable and relatively easy, while providing extensive information on the species present in the area. Changes in the diet of otters could indicate population declines or the introduction of invasive species to an area (Barrientos *et al.*, 2014). Additionally, knowing what an otter is consuming has been informative about changes in the environment, especially after anthropomorphic disturbance to regions (Romero, Guitián and Ruiz-Olmo, 2012).

Otter diet has been studied extensively in the past using morphological analyses of spraints. However, there are significant issues with these traditional diet studies, namely that can only include prey species that leave recognisable remains in spraint (e.g. bones, scales, feathers, shells) (Carss and Parkinson, 1996). Morphological approaches also have issues with identification errors with an inability to get down to species or even family taxonomic levels. Many studies using morphological methods to identify prey have shown a worryingly high error rates in identification when mock faecal samples were created by researchers (Carss and Elston, 1996; Harper *et al.*, 2020). Such studies often produce their own guides to identification which are bespoke to the study making each dataset unique (Reid *et al.*, 2013). The different criteria for identification between studies makes it difficult if not impossible to compare directly with other datasets. Therefore studies of otter diet on regional and national scales have been rare and attempts

at metanalyses on otter diet have had to exclude the majority of studies published due to methodological differences between studies (Reid *et al.*, 2013; Krawczyk *et al.*, 2016). A further issue with morphological analysis of diet is that it cannot be used to look at the relative contribution of different species to an otter's diet because the collection of remains either cannot be assigned to a specific number of prey individuals or it would be too time consuming to do so (Beja, 1996). Furthermore, the cost of morphological analysis has been found to be high at $300 sample due to the time needed to conduct these studies, and this has limited the scope and scale of such studies (Nichols, Åkesson and Kjellander, 2016).

Comparatively recently, metabarcoding has become a popular tool for analysing diet in many species including otters (Harper *et al.*, 2020; Pertoldi *et al.*, 2021). These have revealed a wide variety of prey items in otter diet and delineated the niche occupied by otters relative to other carnivores such as American mink (Harper *et al.*, 2020). These studies have largely corroborated what we knew about otter diet from morphological analyses, i.e. that fish make up most of the diet, estimated at around 80%, while the remainder is made of amphibians, birds, and mammals (Krawczyk *et al.*, 2016). However, morphological studies have often highlighted the important role that invertebrates, such as crayfish and crab, have in the diet of *Lutra lutra* (Brzeziński *et al.*, 2006; Clavero, Prenda and Delibes, 2006). Metabarcoding studies have left invertebrates unexplored, and this is a significant lacuna, as many invertebrates that otters have been observed preying upon (e.g. beetles) are difficult or impossible to identify morphologically. Metabarcoding offers the hope of identifying and quantifying the relative contribution of invertebrates to otter diets, giving us a more complete picture of where otters sit in the trophic networks they occupy (Jang-Liaw, 2021). Furthermore, studies that have included invertebrate species in their investigations diet of *Lutra lutra* have not had a broader marker that encompasses the whole diet. In the absence of a marker which encompasses all dietary items, it is impossible to determine the relative contribution of some taxonomic groups within the diet, i.e. invertebrate and vertebrate.

A major advantage of metabarcoding is the relatively fast production of data and the potential to analyse thousands of samples on a regional, or even international, scale. In fact, the larger the study, the lower the cost per sample. (Deiner *et al.*, 2017). This provides an opportunity to collect samples and account for seasonal and annual cycles in otter diet, something difficult with morphological analysis as they are limited by the time

availability of skilled taxonomists to identify the items in the spraints individually (Carss and Parkinson, 1996). Furthermore, the sensitivity of metabarcoding is far greater, giving us the ability to identify small species or species that do not leave behind hard parts and so are missed with other methods (Massey *et al.*, 2021). This has allowed metabarcoding studies to identify jellyfish as well as molluscs in the diet of other predators. Despite the clear advantages, metabarcoding does have some limitations. Large amounts of host DNA in dietary studies can be an issue in detecting the true diet. This can be remedied by using blocking primers, but this in turn can have impacts on the detection of prey taxa closely related to the predator (Piñol *et al.*, 2015). The choice of marker has a huge impact on which species are identified, as some markers have more taxonomic resolution or may not amplify certain species, leading to incorrect species richness estimates and unreliable abundance estimates. (Alberdi *et al.*, 2018). Other issues with metabarcoding include contamination, tag jump, and poor sample preservation (Zinger, Bonin, Inger G Alsos, *et al.*, 2019).

The few existing studies on *Lutra lutra's* diet in the UK that have used metabarcoding have exclusively focused on the vertebrate aspect of the diet. However, several morphological studies indicate the importance of invertebrate communities, especially crayfish, for otter diet in certain habitats (Delibes and Adrian, 1987; Breathnach and Fairley, 1993). Here, we aim to use a hierarchical marker strategy that provides insight into the breadth of otter diet in Suffolk with a broad Eukaryotic marker to allow the relative contribution of invertebrate and vertebrates to the diet, while also using two more specific markers, a 12s Vertebrate and 16s Invertebrate marker, to retain higher taxonomic resolution in these two groups.

Methods

Sample collection

Eurasian Otter faecal samples were collected from four rivers across Suffolk, UK (River Waveney, Blyth, Ade, Minsmere), based on knowledge of otter locations provided by the Suffolk Otter Group, with collections taking place from 2018 to 2021. The samples were collected by systematically searching both sides of the river and opportunistically collecting spraint when present. The location (using a GPS), date and time, and collector

were recorded for each spraint sample. Locations of sampled sites are shown in Figure 4.1. A total of 411 samples were collected across the four locations (Waveney n=40, Blyth n= 153, Alde n=125, and Minsmere n=93). The whole spraint was collected and placed in a 50 ml falcon tube containing 30 ml of NAP buffer to preserve the faecal sample. At the end of each collection day, all samples were stored at -20 °C until DNA extraction. Field negatives were taken using falcon tubes that contained only buffer at each sampling site. These were shown to contain negligible amounts of DNA, but all controls were sequenced regardless of DNA concentration. Of these 411 samples, 286 were taken forward for sequencing based on the quality and quantity of DNA extracted. Metadata for all samples is present in Supplementary Data 4.1 Table S13. Our collections included tidal, inland and coastal areas, and we recorded this information for each sample (hereafter referred to as the factor 'tidal environment'; tidal n = 116, inland n = 110, coastal n = 41).

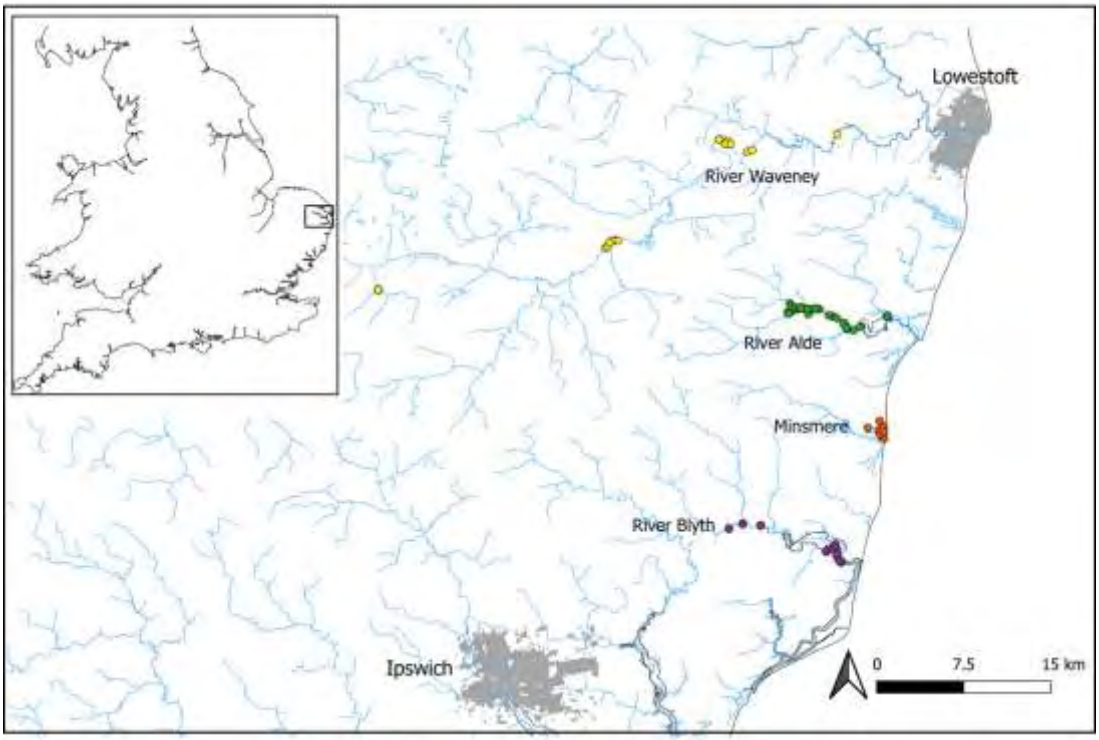

Figure 4.1: Locations for Lutra lutra, Eurasian Otter, faecal sampling in the four rivers, Suffolk, UK.

DNA extraction

The DNA was extracted from the faecal samples using a phenol chloroform method. 0.5g of each faecal sample was washed with 1000 µl of TE buffer and vortexed, centrifuged down at 8000xg for 2 mins. The supernatant was poured off and the process was then repeated. 1000 µl of TNE buffer with 0.5% SDS was added to the sample with 20 µl of proteinase K. The samples were then incubated at 56˚C, vortexed every half hour for the first 2 hours, and then left overnight. After incubation, the samples were vortexed and then centrifuged at 8000xg for 2 mins and 700 µl of the lysate was removed and placed in a fresh 2 ml eppendorff tube. 700 µl of 25:24:1 phenol chloroform isomyl alcohol was then added, and the samples mixed gently for 3 mins. The samples were then centrifuged for 5 mins at 10000xg, and the aqueous layer removed and placed in a new eppendorff tube. An equal volume of 24:1 chloroform isoamyl alcohol was added and the solution was then mixed gently for 2mins. The homogenised sample was then centrifuged for 5 mins at 10000xg and the aqueous layer removed and placed in a new tube to which 40 µl of 5M NaCl was added, as well as 900 µl of 100% ethanol. The solution was gently mixed and then left at -20 ˚C overnight. The next day the samples were centrifuged at 10000xg for 30mins at 4˚C and the ethanol poured off. 900 µl of 70% ethanol was then added to the sample and the sample was vortexed briefly to dissolve any excess salt. The sample was again centrifuged at 10000xg for 30mins. The ethanol was then poured off and the excess ethanol allowed to evaporate off in a sterile PCR hood. The sample was then eluted with 200 µl of AE buffer by incubating it for 10 mins at 56˚C. All samples were then quantified using a nanodrop and normalised to 10 ng/µl using TE buffer.

After extraction and normalisation, we selected 286 samples based on the quality and quantity of the DNA obtained using spectrophotometric readings of a Nanodrop (Thermo-Fisher Scientific) with samples closes to 1.8 rations for 260/280 and 2.0/2.2 for 260/230nm ratios chosen for further analysis. We used a design based on that outlined by Taberlet *et al*. (2018). Each sample was split into three technical replicates, and these were arranged randomly within a matrix that consisted of four 96 well plates. Random assignment to plates was stratified so that in each plate there were five positive controls and five negative controls. Blanks were arranged in a consistent systematic pattern with 12 blanks on each plate arranged so that there was at least one blank present on every row and every column.

Marker Choice and PCR conditions

The markers used for analysing the diet were vertebrate, invertebrate and Eukaryotic markers, shown in Table 4.1. Blocking primers, OBS1 for vertebrate and MamMAVB1 for invertebrate, were used for the 18s and 16s markers at a ratio of 10:1. The optimum cycle number and annealing temperature were determined experimentally by qPCR using cyber green bio line taq. For each marker, a serial dilution of undiluted, 0.5 and 0.1 dilutions was conducted to determine the optimal sample concentration. The final PCRs were amplified with Amplitaq gold 360 master mix (Thermo Fisher Scientific) in a reaction volume of 10 µl. The precise conditions for each marker were: 18s Eukaryotic marker - 95 ˚C degrees followed by 30 cycles of 95 ˚C for 30 seconds, 50 ˚C for 30 seconds and 72 ˚C for 30 seconds, followed by a final extension of 15 mins at 72 ˚C. For the Vertebrate marker, the conditions were 95 ˚C followed by 40 cycles of 95 ˚C for 30 seconds, 48 ˚C for 30 seconds, and 72 ˚C for 30 seconds, followed by a final extension of 15 mins at 72 ˚C. For the invertebrate marker, the conditions were 95 ˚C followed by 45 cycles of 95 ˚C for 30 seconds, 48 ˚C degrees for 30 seconds, and 72 ˚C for 30 seconds followed by a final extension of 15 mins at 72 ˚C. All primers were used at 0.2 µM concentrations and blocking primers were used at a final concentration of 2 µM. After amplification, all samples were checked on 1% agarose gels to ensure successful amplification.

Table 4.1: The markers and blocking primers used in this study.

Marker name	Target taxa	Region amplified	Forward sequence	Reverse sequence	Reference
12sV5	Vertebrates	12s	TTAGATACCCCACTATGC	TAGAACAGGCTCCTCTAG	(M De Barba *et al.*, 2014)
16sMAV	Invertebrates	16s	CCAACATCGAGGTCRYAA	ARTTACYNTAGGGATAACAG	(M De Barba *et al.*, 2014)

MamMAVB1	Mammals	NA	CCTAGGGATAACAGCGCAATCCTATT-C3	NA	(M De Barba *et al.*, 2014)
Euka-02	Eukaryotes	18s	TGGTGCATGGCCGTTCTTAGT	CATCTAAGGGCATCACAGACC	(Guardiola *et al.*, 2015)
OBS1	Lutra	NA	CTATGCTCAGCCCTAAACATAGATAGCTTACATAACAAAACTATCTGC-C3	NA	(Kumari *et al.*, 2019)

Library preparation and sequencing

Each technical replicate had two 8 base pair tags that differed on the forward and reverse primers. These tags were designed using the oligotag software (Coissac, 2012). This allowed all samples to be uniquely identifiable post sequencing. After the initial PCR, all samples were identifiable by their unique tag combination and were pooled into one Eppendorf tube. Each marker was pooled separately and assigned a different illumina adapter, meaning there were three separate libraries that went forward into the library preparation stage. We used the Truseq PCR free kit (*Illumina*)to prepare the libraries following a protocol we developed (see Chapter 2). The samples were run on two separate sequencing runs, one a Miseq V3 2x300bp flow cell for the Eukaryotic marker and a Nextseq mid output 2x150bp flow cell for the invertebrate and vertebrate markers.

Data analysis

The samples were bioinformatically cleaned and analysed using the OBITools package (Boyer *et al.*, 2016) using the python programming language. First, the samples were aligned using illuminapairedend function and the aligned reads were then demultiplexed using ngsfilter, allowing for no mismatches in the tag sequences and one mismatch in the primer sequences. The samples were then dereplicated using obiuniq and mOTUs with

fewer than 5 copies and shorter than 100bp were filtered out of the data set using obigrep. The samples were then dereplicated and taxonomically assigned using the ecotag function. Our reference database was constructed from the EMBL ref database (release March 2022) (https://www.ebi.ac.uk/) and the final database constructed as shown in OBITools3 (Boyer *et al.*, 2016), resulting in a final reference database containing 51,417 unique species for the eukaryotic marker, 46,159 for the invertebrate marker and 20,003 sequences for the vertebrate marker. Only samples that matched at 95% or higher were kept after assignment. The datasets were then reduced to include only taxa relevant otter diet, e.g. the eukaryotic dataset was filtered to only include metazoans. The resulting taxonomically assigned sequences were then imported into R version 4.0.3 and analysed with the metabaR package (Zinger *et al.*, 2021), where the remaining reads were filtered to remove contamination, the specific contaminants present in the negatives were identified for all types of control (extraction, sequencing and PCR) the samples were then screened for these contaminates and PCRs where more than 10% of the reads were identified as contaminants were removed before downstream analysis. Tag jumps were removed using the tagjumpslayer function in metabaR which reduces the abundance of MOTU's relative to theiraverage across the entire dataset as fist proposed by (Esling, Lejzerowicz and Pawlowski, 2015) Finally, the replicates were then bioinformatically pooled into individual samples, and the resulting composite samples were analysed for ecological differences.

Statistical analyses were performed in R version 4.0.3. The coverage of each sample was calculated using Hill numbers in the Metabar package (Zinger *et al.*, 2021). Subsequently river location coverage was also estimated using the iNEXT package (Hsieh, Ma and Chao, 2016). Diet richness was assessed using frequency of occurrence (F_O%) (Liu *et al.*, 2020b). Differences in species richness between the sites were tested using Fisher-pitman permutation tests (Coin *et al.*, 2000). We calculated relative read abundances to assess differences in diet composition while accounting for the differences in read count between samples with the decosatand function in the vegan package (Dixon, 2003)(Deagle *et al.*, 2019). We calculated Bray-Curtis dissimilarity distances with respect to the different river locations with the K=3 and 999 iterations with *metaMDS* function (Bray and Curtis, 1957). A PERMANOVA (Oksanen, 2008)(Anderson, 2001) was used to investigate the effect of tidal environment (coastal, inland, tidal) and river on the composition of samples. We used the adonis2 function in vegan with 999 permutations and tested the data to ensure the assumptions for a

PERMANOVA were fully met using the betadisper function (*VEGAN, A Package of R Functions for Community Ecology on JSTOR*, no date). Our data was visualised using an NMDS (nonmetric multidimensional scaling) and plotted using ggplot2. The envfit function with 999 permutations was used to identify potential species that could be driving the compositional differences between the samples. Further to this, we investigated if there were any species driving the differences found in the data using the indicspecies package (De Cáceres and Legendre, 2009).

Results

Final data set description

After identification and quality filtering, 19,248,301 reads remained for the invertebrate marker, then following removal of chimeras, tag jump and contamination, there was a final dataset of 8,907,543 reads over 286 samples, with 23,724 reads per sample on average. For the vertebrate marker, 16,948,882 reads remained after quality filtering and identification and subsequent removal of non-specific markers tag jump chimeras left a final dataset of 9,768,035 reads, with 38,676 per sample on average. For the Eukaryotic marker, out of an initial 7,453,153 reads following removal of chimeras, tag jump and contamination there were 4,118,396 reads remaining, with an average of 15,030 per sample. Rarefaction curves show all samples except one reached asymptotes; the one samples that did not reach asymptote was filtered out due to low sequencing depth.

Eukaryote markers

Dietary richness

We identified 525 unique mOTUs, 100% to Kingdom, 99.7% to Phylum, 99% to Class, 91% to Order, 73% to Family, 50% to Genus and 36% to species level. The Eukaryotic marker identified 14 phyla in total, with Chordata being present in 97% of samples, Nemotodea in 96%, Arthropodea in 83%, Platyhelminths in 67%, Cnidaria in 37%, Annelida in 18%, Acanthocephala 17%, Rotifera in 4%, Nematomorpha 3% and Tardigrada 2% (Supplementary Data 4.1 Table S4).

On average, we found 72 unique Eukaryotic MOTU's in each faecal sample (SD ± 29, range 16–136). The richness found in the individual faecal samples varied between the sites sampled, with the Waverny being most diverse 96 (SD ± 22, range 50–135) with MOTUs being the least diverse with Minsmere being least diverse 62.9 MOTUs on average (SD ± 19, range 21–136). A full breakdown of diversity of each site is available in (Supplementary Data 4.1 Table S2).

Dietary abundance

The dietary abundance data largely corresponds to the dietary richness data. For the Eukaryote marker, Chordates were the most abundant phylum in the data constituting 22% of total reads, Nematodes 21%, Arthropods 18%, Platyhelminthes 15%, Cnidaria 9%, Acanthocephala 5%, Annelida 5%, Rotifera 2%, Echinodermata 1%, Gastrotricha 1%, Mollusca 1% and Tardigrada 1% (Figure 4.2A; Supplementary Data 4.1 Table S6). Although the patterns of abundance are largely similar to the frequency of occurrence data, the relative abundance of arthropod sequences is higher than would have been expected, becoming the second most abundant phylum. Of the above taxa, only Chordates, Arthropods and Mollusca are relevant to otter diet, only these taxa are considered as to contribute to a proportion of the diet in subsequent analysis of diet (Figure 4.2B). A perMANOVA test found significant differences among rivers in otter relevant taxa (df = 4, r squared = 0.04, F =2.26, p value = 0.001), with greater dispersion in the Alde compared to the Waveney (Figure 4.3A).

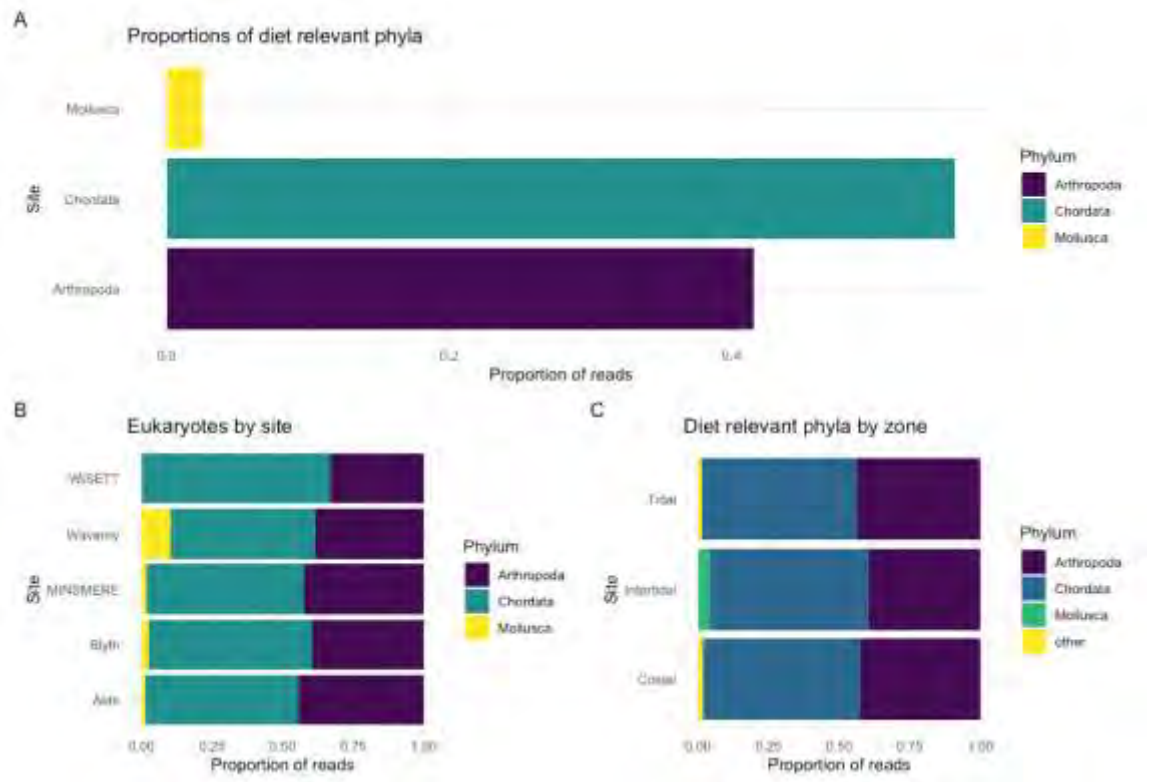

Figure 4.2: A) The proportions of relevant phyla revealed by the Eukaryotic marker across the entire dataset. B) the proportions of all phyla that comprised more than one percent of reads broken down by site C) the proportions od diet relevant phyla broken by ecological niche .

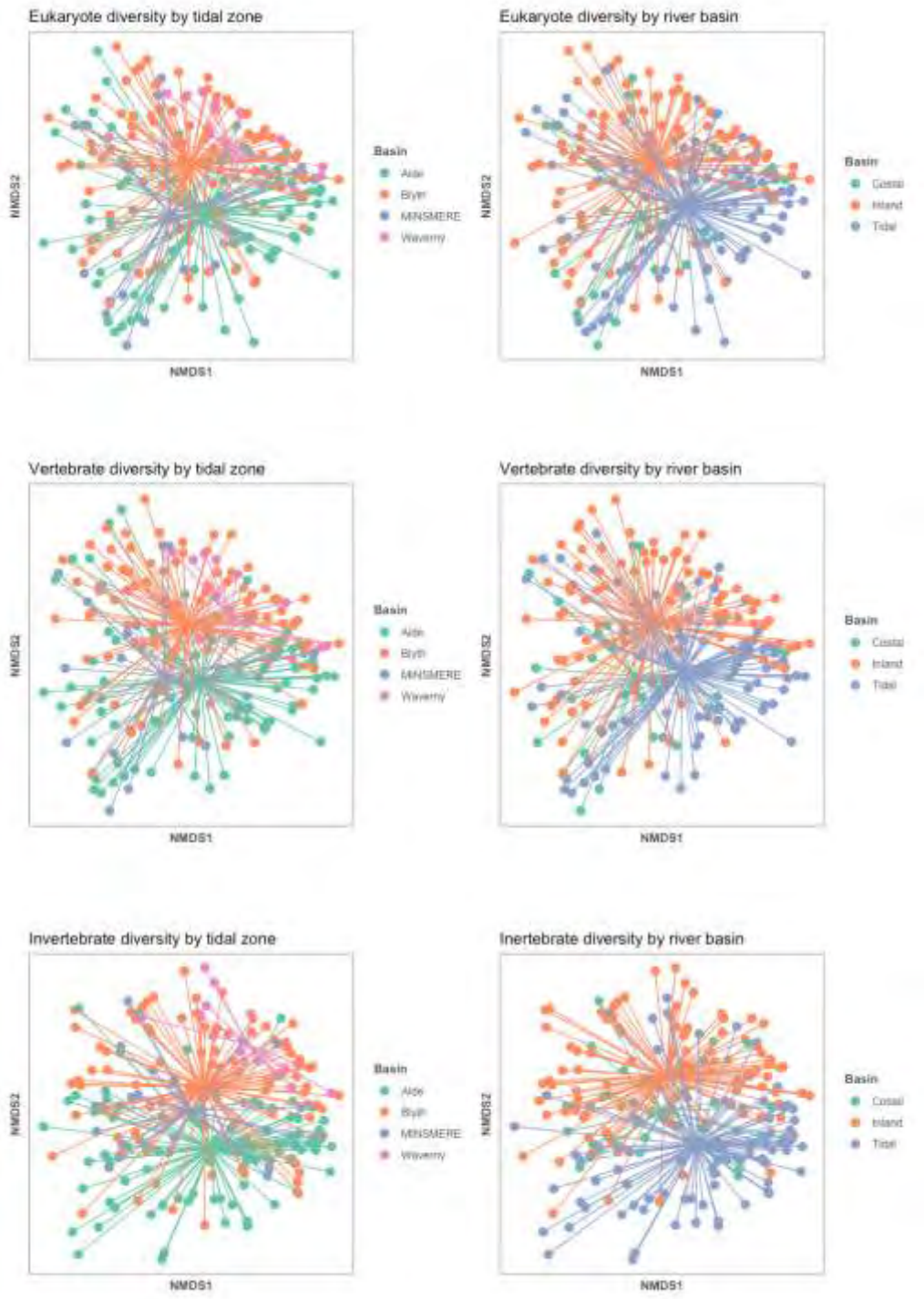

Figure 4.3 :Non-metric multidimensional scaling plots for each marker by site and by tidal zone, A) The eukaryotic taxa relevant to otter diet by river location . B) The relevant eukaryotic markers broken down by tidal zone. C) The vertebrate taxa diversity broken down by site, D) The vertebrate taxa diversity broken down by tidal zone, E) The relevant invertebrate taxa by site, F) The relevant invertebrate taxa marked by tidal zone.

Vertebrate marker

The blocking primer included in our assay kept host DNA down to less than 1% of the sequences, successfully increasing the reads available for the true diet, aiding in sequencing depth. We found 164 unique sequences of which we identified 100% to Class, 98.5% to Order, 94.1 to Family, 59.1% to Genus and 32.2% to species level (Supplementary Data 4.2 S1). We considered all the vertebrate species identified to be realistic prey items for the otter and so all species were included.

Dietary richness

On average, we found 17.8 unique Vertebrate MOTU's in each faecal sample (SD ± 6.01, range 5–35). The richness found in the individual faecal samples varied between the sites sampled, with the Waverny being most diverse 18.7 (SD ± 6.44, range 6–32) with MOTUs being the least diverse with Blyth being least diverse 16.8 MOTUs on average (SD ± 6.2, range 5–35). A full breakdown of diversity of each site is available in (Supplementary Data 4.3 Table S2)

MOTU data was turned into presence absence for each sample thereby excluding abundance information and frequency of occurrence calculated by site and habitat type. There were 38 families that were present in 1% or higher (Supplementary Data 4.2 Table S4). The most common families by frequency in the spraint were Gasterosteidae (sticklebacks) present in 61% of samples, followed by Gobiidae (Gobys) 37%, Anguillidae (eels) 28%, Anatidae (ducks) 24%, Leuciscidae (minows) 23%, Nemacheilidae (stone loaches) 23%, Tincidae (Tench) 21%, Pleuronectidae (dabs) 15%, and Ranidae (frogs) 10%. All other families were present in less than 10% of samples. The dietary richness present in each river basin varied significantly (Fisher-Pitman Permutation Test, chi-squared = 37.195, p-value < 0.001). However, in all river basins sticklebacks were the most frequent prey item. The most common non-stickleback prey item in the Waveney was roach, while eel was commonly found in Blyth samples, and Tench was important in the Alde basin (Supplementary Data 4.2 Table S3). There were also differences between costal, inland and tidal zones sampled (Fisher-Pitman Permutation Test, chi-squared = 8.5177, p-value = 0.0337) Gobies were the most common group in the tidal zone, present in 58% of samples, while sticklebacks were the

most common in inland and costal zones, present in 78% and 57% of samples, respectively. Stone loaches were more commonly present in inland areas, present in 42% of samples, compared to 6% in tidal areas. Eel was present in 33% of samples from tidal areas compared to 25% of inland samples. Tench was important in coastal areas (49% of samples) compared to 21% and 10% of tidal and inland zones, respectively.

Dietary abundance

The relative read abundance data revealed that fish comprised of 79% of the vertebrate portion of the diet, birds 13%, mammals 5% and amphibians 3% (Figure 4.4). The most common families identified were Gasterosteidae (sticklebacks) comprising 15% of vertebrate reads, Gobidae (gobies) 10%, Anatidae (ducks) 10%, Anguiilidae (eels) 9%, Tincidae (Tench) 8%, Nemcheilidae (stone loaches) 7%, Leuciscidae (minnows) 6%, Pleuronectidae (dabs) 6% and Cricetidae (voles) 4%. All other species comprised of 3% or less of the relative vertebrate abundance (Supplementary Data 4.2 Table S6). The relative abundances show some key differences in comparison to the frequency of occurrence data. Ducks were of greater importance in abundance than in frequency of occurrence, being the joint 2nd most abundant family, while voles and dabs were important in the relative abundances despite being negligible in frequency of occurrence data.

There were significant differences in our abundance data between river basins (perMANOVA, df = 4, r squared = 0.04, F =2.26, p-value = <0.001), with the Waveney being a subset, thus less diverse, than the others (Figure 4.3C). There were also significant differences between tidal, inland and coastal zones (df = 1, r squared = 0.04, F =2.26, p-value = 0.002), with inland differing from tidal, and coastal overlapping with both (Figure 4.3D). Sticklebacks were the most common family for all river basins at 12%, except for Minsmere where the most abundant family were gobies (12% of reads). The otter spraints in the Blyth basin and Minsmere had more eel with 9% of vertebrates reads belonging to that species in both sites, while cyprinids were more common in the Waveney (11% of reads in total) (Supplementary Data 4.2 Table S5). Tench was abundant in vertebrate reads all locations, with around 5-10% of vertebrate reads in each. The species indicator analysis showed 30 taxa that were significantly associated with one

of the sites, 20 to the Waveney, six species to the Alde, three to the Blyth, and one to Minsmere (Supplementary Table S1).

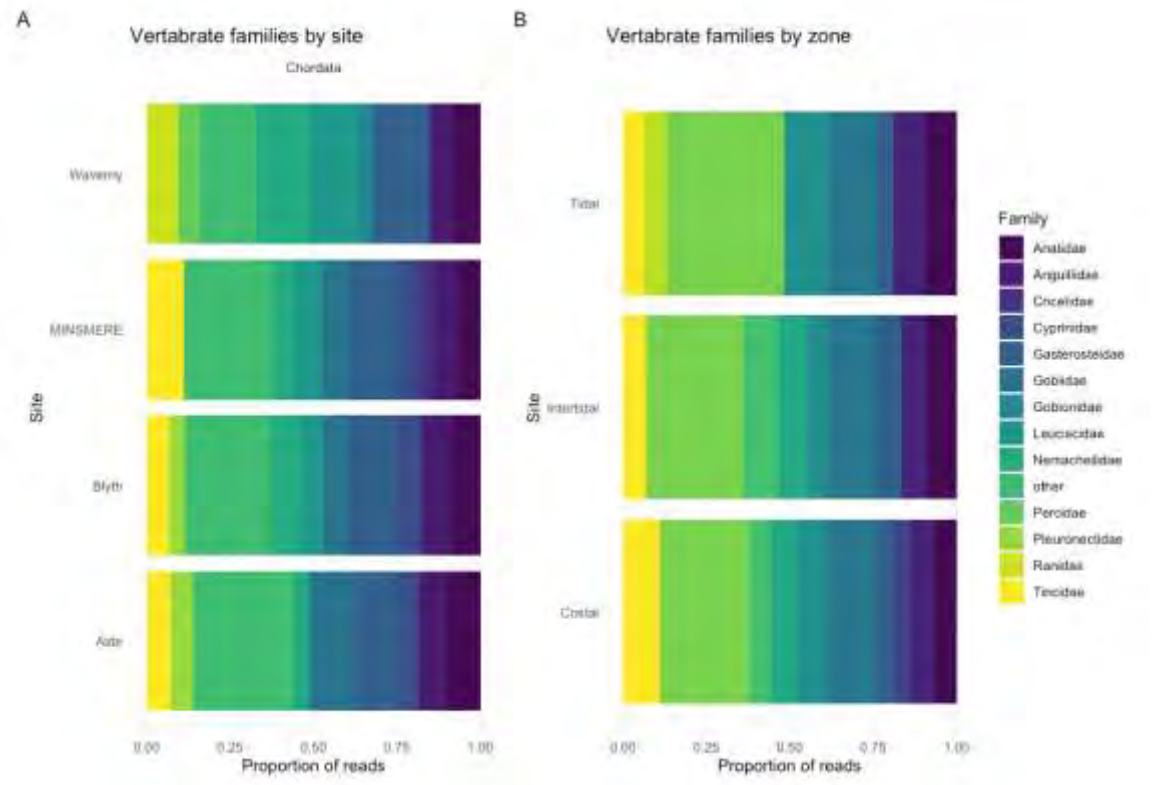

Figure 4.4: showing the importance of different functional groups in the vertebrate portion of the diet with fish making up 79% of reads, Birds 17% mammals 5% and amphibians 3%. The second ring breaks those functional groups down by family and the final outer inf by genus or species depending on the level of identification.

The most common family did not differ between tidal environments, with sticklebacks being the most abundant family in all tidal environments, making up 14-15% of vertebrates read abundance in each zone. Eel was more important in tidal zones than in inland and coastal sites, with 10 % of tidal zone reads being assigned to eel compared to 7% in the inland and coastal sites. Tench was important in coastal areas with 11% of reads, compared to 6% and 7% in tidal and inland. Stone loaches were important in inland areas (11%), compared to coastal (6%) and tidal (4%) (Supplementary Data 4.2 Table S9). The species indicator analysis showed 16 species that were significantly associated with one tidal environment; six to coastal zones, six to inland and four to tidal zones (Supplementary Table S2).

Invertebrate marker

We used a mammal blocking primer to reduce the amount of non-specific amplification. Despite this, the invertebrate marker had issues with amplification of bacterial and unidentified reads that comprised 66.4% of the data. However, rarefaction curves show the remaining reads were sufficient to gain full coverage of invertebrate alpha diversity in almost all samples. After bioinformatic cleaning and aggregation of reads into unique species, we found 1023 species in total. Of those species we identified 100% to Kingdom, 98.9% to Phylum, 92.6% to Class, 76% to Order, 62.5% to Family, 53.4% to Genus, and 42.5% to species (Supplementary Data 4.3 Table S1). Subsequently we only analysed taxa from Phlya that were considered relevant to the otter diet, which comprised arthropods and molluscs.

Dietary Richness

On average, we found 28.7 unique invertebrate MOTU's in each faecal sample (SD ± 13.02 , range 0–70). The richness found in the individual faecal samples varied between the sites sampled, with the Waverny being most diverse 40.22 (SD ± 11.9, range 21–59) with MOTUs being the least diverse with Minsmere being least diverse 23.5 MOTUs on average (SD ± 13.2, range 2–64). A full breakdown of diversity of each site is available in (Supplementary Data 4.2 Table S2)

The most frequently occurring diet-relevant families of invertebrates were Gammeridae, which were present in 22% of samples, Palaemonidae (shrimp) present in 17%, Carcinidae (crab) 11% and Crangonidae (shrimp) 10%. Staphylinidae (rove beetles) were present in 6% of samples (Supplementary Data 4.3 Table S4). The majority of Palaemonidae were identified as glass shrimp, while Crangonidae reads were found to belong to the genus Crangon, which contains several commercially important species. The 337 other families identified in the invertebrate dataset families we classified as not relevant as they would not have constituted anything large enough for *Lutra lutra* to consume directly (Supplementary Data 4.3 Table S4). The most common family

excluded was the water roaches (Asellidae), followed by midges, neither of which would be direct prey items.

The invertebrate communities did vary significantly between river basin (one-way Fisher-Pitman Permutation Test, chi-squared = 19.85, p-value < 0.001) or between tidal environments (one-way Fisher-Pitman Permutation Test, chi-squared = 12.4, p-value = 0.005). Of the relevant invertebrate taxa, *Gammaridae* (shrimp) were found in 56% of samples from the Waveney and 26% of samples from the Blyth, while Palaemonidae were present in 34% of samples from Minsmere compared to 0-10% at the other sites. While Carcinidae (crab) was present in 20% of samples from Minsmere and 10% of samples from the Blyth. Crangonidae was also present in 10% of samples from the Blyth and 6% from the Waveney. Beetles including rove (*Staphylinidae*) and ground beetles (Caribidae) were present in all our sites although at frequencies of less than 10% in each (Supplementary Data 4.3 Table S3). The invertebrate frequencies varied between tidal zone for prey species. Carcinidae (crab) was present in 19% of samples on tidal areas, but only 4% from inland areas and was not present in samples from costal samples. The Palaemonidae family (shrimp) were most abundant in coastal areas 34% of samples while they only constituted 23% and 5% respectively for tidal and inland areas.

Abundance

The most common invertebrate prey families based on relative read abundances were Gammaridae 5%, Palaemonidae 4%, Crangonidae 3%, all species of shrimp (Figure 4.5A). *Carcinidae* (Crab) made up a further 3% of reads. All other potential prey families made up less than 2% of the total invertebrate read abundances (Supplementary Data 4.3 Table S6). There were significant differences between the sites in phlya (perMANOVA, df = 4, r squared = 0.02, F =2.3, p-value <0.001), with the Waveney being less dispersed than other rivers (Figure 4.3E). Crab making up 4% of reads in the Alde, 3% in the Blyth, 2% in the Waveney and 1% in Minsmere. While shrimp abundances also varied, with the Palaemonidae family constituting 9% of reads for Minsmere but less than 5% for any other site (Supplementary Data 4.3 Table S5). There were also significant differences in the phyla between the tidal environments (perMANOVA, df = 1, r squared = 0.014, F =3.2, p-value <0.001; Figure 4.3F). *Carcinidae* (crab) was present in 5% of tidal samples but only 2% of inland or coastal samples. Gammaridae (shrimp) constituted

5% of tidal and inland samples, and 3% of coastal. Palaemonidae was most common in coastal (9% of samples) compared to 5% in tidal and 3% of inland sample reads. Species indicator analysis found 21 species associated with one site and 13 species associated with one tidal zone; however, none of these species were diet relevant (Supplementary Data 4.3 S11 and S12).

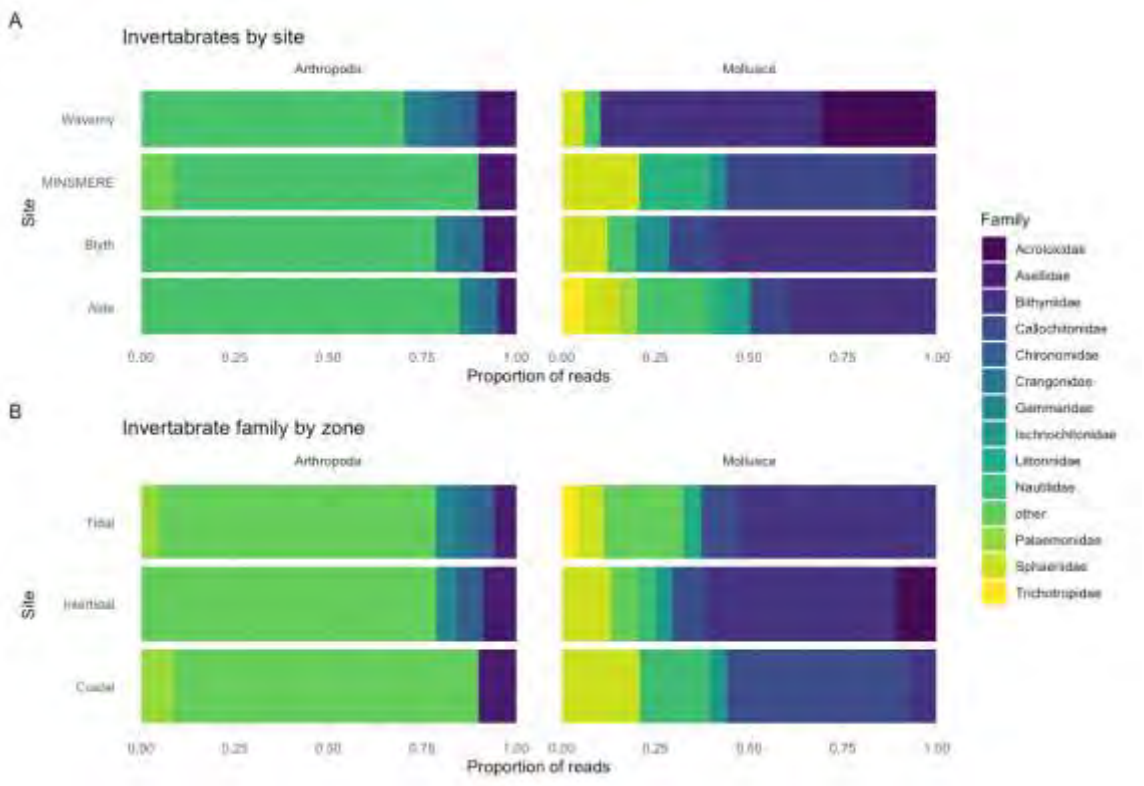

Figure 4.5: The relative proportions of invertebrate A) The proportions of all relevant invertebrate taxa broken down by site. B) The proportions of diet-relevant invertebrate taxa broken down by ecological niche.

Overall diet

Overall from the Eukaryote marker, we found that 53% of abundance was vertebrates, once non-metazoans were removed, and 47% was invertebrate reads. We consider that 100% of the vertebrate taxa were relevant to the diet, but only 18% of the invertebrate. This means that invertebrates conservatively make up 8.3% of the diet in terms of abundance. This is likely to be an underestimate because we know that this invertebrate marker has been less reliable when giving relative abundances (Chapter 2). Frequency of occurrence data show that diet-relevant invertebrate taxa occurred in 6-22% of samples, which may indicate that they are consistently consuming these items.

Discussion

Using the combination of broad and specific markers allowed us to assess that at least 8% of Eurasian otter diet comprised arthropod. This may be at the lower end of the contribution of arthropods to diet due stringent filtering of the data. Birds have previously been estimated to be 3% of otter diet (Harper *et al.*, 2020) and usually considered in diet studies. Our finding that invertebrates are as least as important highlights the importance of encompassing the whole diet of a predator rather than just focussing on the taxa considered to be most important. The importance of invertebrates has been spoken about in a number of traditional morphological studies (Clavero, Prenda and Delibes, 2006; Melero *et al.*, 2008; Krawczyk *et al.*, 2016) however, metabarcoding studies have largely ignored this facet of the diet. The population of otters we have studied have been the subject a traditional morphological analysis over several years, which included over 1000 samples (Suffolk Otter Group, unpublished data). Our analysis revealed both a greater breadth of diet and at a higher taxonomic resolution than the morphological study

Marker selection was key to the success of our study. The Eukaryotic marker was able to give an overview of the relative importance of vertebrate and invertebrate families in the diet and the higher specificity of the vertebrate and invertebrate markers allowed for a higher level of identification then would have been possible with one marker alone. The Eukaryotic marker provided a general outline of the diet and identified Chordates as the most common taxa (22%) followed by Arthropoda (19%) of reads, allowing us to

contextualise the relative importance of vertebrates and invertebrates. In the absence of the eukaryotic marker it would be easy to conclude that invertebrates are more important in the diet than they are. The impact of marker choice has been extensively studied and remains difficult with a compromise between several factors including cost, reference database quality and amplification bias (Chapter 2) (M De Barba *et al.*, 2014; Stefanni *et al.*, 2018; Casey *et al.*, 2021a). Despite high identification rate achieved in this study more specific markers such as for fish and arthropods would have been advantageous. Future studies would also need to tailor their approach to match markers to the expected diet of otters in a given area.

As expected, fish were the main contributor to the diet (72% of all diet) and that agrees well with previous work (Reid *et al.*, 2013; Krawczyk *et al.*, 2016). However, birds made up 12% of diet in our study, with ducks being the largest component, which is somewhat higher than what has been reported elsewhere. A recent meta-analysis of *Lutra lutra* diet across Europe assessed that birds make up around 3% of otter diet (Harper *et al.*, 2020). Surprisingly we identified geese as a species the otters were preying upon, although this supports anecdotal evidence. The relatively high proportion of birds in the diet may indicate diversification of diet, which can occur due to low availability of favoured prey species (Dettori *et al.*, 2022)or could specialisation of this population for predating on birds. The rivers in the study are fairly small and this may contribute to lower fish populations, pushing the otters to diversify their diet, similar to what has been reported in other otter species (Tinker, Bentall and Estes, 2008). Furthermore, the fish in the diet in this study were generally smaller species, such as sticklebacks, and larger species such as Cyprinidae were present in notably smaller proportions. For example, in our study only 7% of fish consumed were carp, less than has been found in recent metabarcoding and morphological studies of otter spraint in other areas (Harper *et al.*, 2020; Pertoldi *et al.*, 2021). Amphibians in our data made up a lower proportion of the diet at 3% rather than the 17% on average in the meta-analysis (Reid *et al.*, 2013. Mammals were more abundant than previous studies have found at 3% of the diet (Reid *et al.*, 2013). Mammals found in our dataset were exclusively rodents; water voles and shrews were the most common group followed by mice.

When using the invertebrate marker, we identified shrimp and crab as common prey items. Carcinidae (crab) was well represented in the tidal zone constituting 5% of reads in those samples and it was present in 19% of tidal samples. All reads identified as

Carcinidae were identified to the genus *Carcinus,* which contains the common crab, ubiquitous on the UK coastline. Palaemonidae and Gammaridae (shrimp) made up 8% of the reads in the invertebrate dataset. There were several species level identifications, however one species, *Crangon hakodatei* would have placed the shrimp outside its native range, suggesting misidentification either due to marker resolution problems or incomplete databases. The other shrimp species identification (*Crangon crangon*) is a commercially important species in the UK. The relatively high diversity and abundances of arthropods underlines the need to include invertebrate markers in otter diet metabarcoding studies in the future.

We expected to find differences in the diet diversity present between sites, as *Lutra lutra* is well known to be very plastic in its diet composition, and we found that otter diet varied between rivers and tidal zones. We found otters in the Waveney had more larger fish species in their diet than the other sites in this study, including Chubb and Perch, and diet was more diverse in general than the other river systems, which is not surprising as it is a larger river than the other sites. Eel was proportionally more common in the Blyth and Minsmere sites than in the Waveney and Alde, indicating those rivers may be important reserves of eel populations which are known to be in decline generally (Castonguay and Durif, 2016). We also found that diet changed in the different tidal zones, with species such as flounder and gobies being associated with tidal areas, while Chubb and ducks were associated with inland areas. There were more similarities in terms of diet items between coastal and inland areas, likely because the water is not very brackish in coastal locations, such as Minsmere.

The Hierarchical marker structure allowed for broader conclusions about the diet to be made than would otherwise have been possible giving insight to the relative contribution of invertebrates to *Lutra lutra* in comparison to vertebrates. However, the choices of marker in this study did present some issues. The invertebrate marker amplified many non-target taxa that would not have been consumed by *Lutra lutra* directly, and even those species which could be direct prey are difficult to distinguish from incidental consumption, a well-known issue in metabarcoding studies of this kind. This makes characterising the importance of invertebrates to the diet in this study difficult and future studies may want to choose more specific invertebrate markers to reduce these issues.

Despite this, we find the insights provided by looking into the importance of invertebrate to *Lutra lutra* in these populations worth the difficulties undertaken as conservatively invertebrates are likely to represent 8% of the diet in these populations making them a significant part of the diet.

Metabarcoding contains many stages that can introduce contamination from the field to extraction PCR and library preparation. We addressed these issues with a thorough experimental design following the findings of previous studies (Taberlet *et al.*, 2018; Zinger, Bonin, Inger G. Alsos, *et al.*, 2019; Carøe and Bohmann, 2020). This allowed the isolation and identification of contamination at all these points, so we were able to remove contamination in the appropriate fashion. Post sequencing, we were able to use the various types of control PCR, extraction, sequencing to curate the dataset thoroughly and minimise the impact of negative controls thereby ensuring the accuracy of the data produced despite. Despite choosing three markers that encompassed the diet of *Lutra lutra,* there were still issues identifying many mOTUs to species level, which was caused by the markers not having enough resolution and by incomplete reference databases. Unique mOTUs are still not always identified to species level. Having multiple markers for each taxonomic group my alleviate some of these issues because the weaknesses of one marker can be compensated for by another marker. Further development of reference databases is also essential if metabarcoding studies are to reach their potential.

Our results clearly indicate that DNA metabarcoding provides important insights into otter diet and this agrees with previous assessments (Kumari *et al.*, 2019; Harper *et al.*, 2020). Additionally, we demonstrate that using primers that represent a wide breadth of major taxa, including vertebrates and invertebrates, is essential if the diet is to be accurately assessed. We identified a wide variety of realistic prey items, many of which are new to our knowledge of these populations, despite otter diet having been extensively assessed in many previous studies. Overall, the use of broad as well as specific genetic markers is essential to fully capture *Lutra lutra*'s ecological niche, using hierarchical markers to explore the full breadth of diet. This could potentially help identify more aspects of other mesocarnivore diets in the future (Stefanni *et al.*, 2018; Topstad *et al.*, 2021). Most importantly, we demonstrate invertebrates make up a substantial portion of the otter diet, and this has implications for otter conservation strategies.

Data availability,

All supplementary information files are available in a public repository on Zenodo.com https://doi.org/10.5281/zenodo.8060380 raw sequencing files will be available upon publication.

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Supplementary information

Supplementary Table S1: Indicator taxa that characterised the differences between river basins. Asterisks highlight significant results.

Species	Test statistic	P value
River Alde		
Hemibarbus-Squalidus clade	0.535	0.0008
Microphysogobio tafangensis	0.412	0.0494
Cyprinus	0.378	0.0321 *
Diptychus maculatus	0.378	0.0321 *
Xenocypridinae	0.378	0.0325 *
Bufo bufo	0.378	0.0190 *
River Blyth		
Sturnidae	0.641	0.0134 *
Ciliata mustela	0.512	0.0037 **
Acridotheres cristatellus	0.385	0.0164 *
MINSMERE		
Arvicola amphibius	0.692	0.0276 *
RIVER WAVERNEY		
Pungitius platygaster	0.690	0.0010 ***
Rutilus rutilus	0.618	0.0004 ***
Percidae	0.611	0.0008 ***
Squalius cephalus	0.559	0.0010 ***

Alburnus alburnus	0.522	0.0159 *
Cottus	0.504	0.0106 *
Rana	0.501	0.0039 **
Sander marinus	0.500	0.0022 **
Gymnocephalus cernua	0.500	0.0066 **
Rana wuyiensis	0.442	0.0154 *
Barbatula toni	0.442	0.0301 *
Perca schrenkii	0.433	0.0016 **
Squalius carolitertii	0.433	0.0037 **
Gobio	0.433	0.0056 **
Perca	0.426	0.0031 **
Cottioidei	0.420	0.0099 **
Gobio acutipinnatus	0.354	0.0337 *
Gobio lozanoi	0.354	0.0361 *
Lepidotrigla	0.352	0.0460 *
Lutjanus	0.338	0.0491 *

Supplementary Table S2: Indicator species that characterised the differences between tidal environments. Asterisks highlight significant results.

Species	Test statistic	p.value
Coastal species		
Esox lucius	0.466	0.0151 *
Lissotriton vulgaris	0.356	0.0063 **
Incilius cristatus	0.249	0.0248 *
Inland species		
Gobioninae	0.436	0.0009 ***
Callonetta leucophrys	0.406	0.0197 *
Cyprinus acutidorsalis	0.384	0.0069 **
Squalius cephalus	0.302	0.0185 *
Cottus	0.295	0.0360*
Tidal species		
Pomatoschistus	0.511	0.0007 ***
Glyptocephalus	0.300	0.0495*

Chapter 5 - Using metabarcoding to reveal geographical differences in the diet of the Neotropical River Otter in Guatemala

Abstract

The Neotropical Otter *Lontra logicaudis* is a mesocarnivore with a range that extends throughout South and Central America. Populations are currently in decline across its range, with the species under threat from changes in land use, prey loss and human conflict, amongst other issues. Despite being categorised as endangered within Central America (Lopes Rheingantz and Gallo-Reynoso, 2021), we still know comparatively little about its diet in this region. Here we use a metabarcoding approach of faecal samples (n = 108) to uncover variations within the its diet in four different locations (La Sirena, Rio de la Passion, Sipacate, and Monterico) in Guatemala. We quantify for the first time the relative importance invertebrates make to *L. longicaudis* diet, comprising 15%. The families Portunidae (crab), Palaemonidae (shrimp) and Pseudothelphusidae were the most abundant invertebrate prey items. Fish made up 81% of the vertebrate portion of the diet, with Eleotridae (sleeper gobies) and Loricariidae (armoured catfishes) being the most common prey items. Armoured catfish are non-native and invasive in Guatemala. Diet differed significantly between sites for both vertebrate and invertebrate markers, with patterns following expected biogeographic differences. Our study uncovers a wide variety of prey taxa at higher resolution than previous studies into the diet of Lontra longicaudis have shown. Our study found evidence of non-native invasive armoured catfish outside of their currently described Guatemalan range, demonstrating the potential for diet studies to go beyond just diet, and reveal changes in the surrounding ecosystem that will ultimately have an impact on conservation issues.

Introduction

The world is currently approaching a 6th mass extinction event driven by anthropomorphic actions including deforestation, urbanisation and the introduction of invasive species (Ceballos, Ehrlich and Dirzo, 2017). This has led to terrestrial ecosystems losing up to 20% of their diversity. In a rapidly changing world, it is important to take stock of the remaining diversity and elucidate the trophic interactions that are fundamental to keeping ecosystems functioning and species extant (Schrodt *et al.*, 2019). Diet studies have the potential to play an important role in conservation as diet can inform us about a species' ecosystem function, its interactions with its environment, and its preferred habitat (Monterroso *et al.*, 2019). Predators also give key information about other species present in the ecosystem, and changes in the diet of a predator can be an indication that new species have entered an ecosystem if a species suddenly appears in the diet, or if a species disappears, it could be an indication of a decline in its population (Dale, Adams and Bowyer, 1994). As such, diet studies have been used to investigate the spread and prevalence of invasive species and to determine the prey communities present at sites (Barrientos *et al.*, 2014; Alexander, Skein and Robinson, 2022). Diet studies of multiple carnivores have also been used to investigate competition between species and the cumulative pressure multiple carnivores may have on prey population size and behaviour (Templeton and Shriner, 2004; Falk *et al.*, 2015).

Lontra longicaudis, the Neotropical Otter, is a midsized semi-aquatic carnivore. Its range stretches from Argentina to Mexico (Rheingantz, Santiago-Plata and Trinca, 2017) but, despite its huge range, it is considered threatened and endangered in many countries, including Guatemala. *L. longicaudis'* need for unpolluted rivers places it at risk from human disturbances. It is further threatened by human-otter conflict and by other human activities such as fishing (Barbieri *et al.*, 2012). Where we have data, *L. longicaudis* is known to be undergoing population declines across its range, but the precise causes of decline are poorly understood, and gaining insights into this trend is key to ensuring the species' survival (Rheingantz, Santiago-Plata and Trinca, 2017; Lopes Rheingantz and Gallo-Reynoso, 2021). As a result of these declines, populations have become increasingly fragmented and vulnerable to extinction; this is especially true of Central America, a region of the world undergoing some of the fastest rates of deforestation in

the world (Hoang and Kanemoto, 2021). The elusive nature of *L. longicaudis* makes it difficult to study, and we, therefore, have little knowledge of either its local distribution, population size or diet in Central America (de Almeida and Ramos Pereira, 2017a).

The Neotropical Otter is predominantly a piscivore, with previous morphological studies identifying 90% of the diet as coming from various fish species (de Almeida and Ramos Pereira, 2017a; Rheingantz, Santiago-Plata and Trinca, 2017). It's diet is highly plastic and reflects the local fish diversity, but often consists of catfish and species such as gobies and cichlids. Most of the prey is small in size, as is the case with other otter species (Quadros and Monteiro-Filho, 1999; Mayor-Victoria and Botero-Botero, 2010; Vezzosi *et al.*, 2014). The non-fish vertebrate diet has been observed to include mammals, birds, and amphibians, although these taxa contribute less than 10% on average (Carvalho-Junior, Birolo and Macedo-Soares, 2010; de Almeida and Ramos Pereira, 2017a). Invertebrate species such as crabs have been found to be almost as important as fish in some populations, but identifying the contribution of invertebrates to the diet with any certainty has, up until now, remained elusive (Pardini, 1998). The Neotropical Otter has also been witnessed to forage on fruits when they are easily available, making the diet extremely broad in nature (Quadros and Monteiro-Filho, 1999). To date, all diet information has been determined through morphological studies where spraint is collected and the hard parts of the species consumed, such as bones, scales, and shells, are identified. This methodology, although informative, is open to observer bias, something which has been extensively studied (O'Rourke *et al.*, 2020). Morphological methods are unable to identify species that do not leave easily identifiable body parts post digestion (Carss and Parkinson, 1996) and require skilled researchers to commit a large amount of time to identify all hard parts present in the faecal samples. Even for experienced practitioners, it is often impossible to provide identification to family level, meaning prey types such as invertebrates are often identified only as decapods or crustations (Mayor-Victoria and Botero-Botero, 2010; Silva, Nascimento and Quintela, 2012). The development of next-generation sequencing methodologies presents the opportunity to improve on these studies and provide more accurate identification with higher taxonomic resolution (Harper *et al.*, 2020).

Metabarcoding of faecal samples is a well-established way of characterising a species' diet, having been used for a wide variety of species (Kartzinel *et al.*, 2015; Thomsen and Willerslev, 2015; de Vos *et al.*, 2018). Metabarcoding is used to amplify short sequences

of highly variable stretches of DNA, that can be used to distinguish between species of interest (Pollock *et al.*, 2018). The millions of reads produced by high throughput sequencers allow thousands of samples to be processed in parallel, and the large number of samples that can be processed in one run makes the process very cost-effective (Watts *et al.*, 2019). Allied to this cost-effectiveness is the increased taxonomic resolution metabarcoding can achieve relative to morphological studies, assuming there is a sufficiently representative reference database of prey species (Coissac, Riaz and Puillandre, 2012). The sensitivity of metabarcoding allows for the processing of samples which are not in optimal condition (Thuo *et al.*, 2019). This is a major advantage when studying rare and elusive species, as collecting samples can be difficult, and it can be hard to accumulate enough high-quality samples for morphological analysis. Such advantages make metabarcoding an excellent technique for studying the Neotropical Otter, which is both elusive and present in low densities, making sample collection difficult (Rheingantz, Santiago-Plata and Trinca, 2017).

Currently, there are no diet studies on *L. longicaudis* that have used metabarcoding, although there are several studies that have been undertaken in other otter species (see Chapter 4). The high biodiversity hotspot *L. longicaudis* occurs in, including spanning the Pacific and Caribbean coasts, creates difficulties for traditional diet studies because the prey items on both coasts are very different, requiring higher taxonomic skills to identify all species. Metabarcoding therefore, provides a potential answer as long as the reference database has sufficient coverage. We investigate the relative contribution of vertebrate and invertebrate taxa to the diet of *L. longicaudis* at four sites across Guatemala, Central America, and determine if studying the otter diet with metabarcoding can identify invasive species of catfish in rivers where it is currently unreported.

Methods

Sample collection

Samples were collected from four sites in the summers of 2019 and 2020 permits (DRM/005/2020|DNRO 002/2018). A total of 108 samples were collected from four sites

in Guatemala: Río de la Pasión, Sipacate, Monterico, and Finca la Sirena (Figure 5.1). Río de la Pasión is a large river in the north of Guatemala, and where sampling occurred the area was dominated by cattle pasture. Finca la Sirena is a private reserve of well preserved tropical forest, located on the Río Dulce on the Caribbean side of Guatemala. Sipacate is a mangrove-based river system on the Pacific coast. Monterico is a river on the Pacific coast with a fairly well-preserved gradient of mangrove to reed bed. Rivers at each location were traversed using a canoe, and likely locations of otter latrines were searched for indications of otter activity and spraint. Upon finding a suitable sample, the spraint was placed in a 50ml falcon tube containing NAP preservation buffer. Field negatives (NAP buffer only) were also taken at every sample location and sequenced. All 108 samples were taken forward for sequencing. Sample collection information is presented in the Supplementary Data 5.1 S8). .

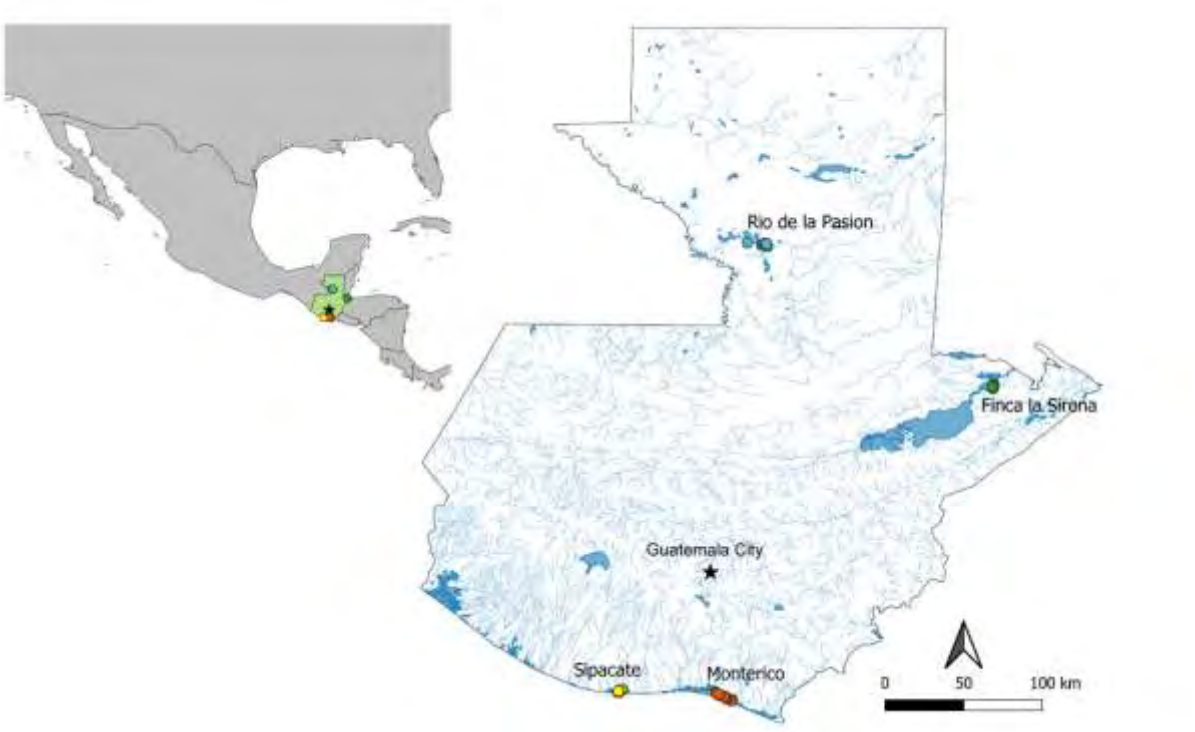

Figure 5.1: Location of sampling points within the four sampling sites for Neotropical Otter, Lontra longicaudis, in Guatemala. The location of Guatemala showing its Caribbean and Pacific coasts is inset.

DNA extraction

The DNA was extracted from faecal samples using the same methodology as presented in Chapter 4. A total of 122 samples were taken forward for analysis (some faecal samples were duplicated due to large volume), normalised and put into a randomised stratified plate design as described in Chapter 4, following Taberlet *et al*. (2018).

Marker Choice and PCR conditions

The markers used for analysing the diet were vertebrate, invertebrate and Eukaryotic markers (Table 1). Blocking primers were used for the 12s and 16s markers at a ratio of 10:1 (Table 5.1). The optimum cycle number and annealing temperature were determined as described in Chapter 4. The final PCRs were amplified with Amplitaq gold 360 master mix (Thermo fisher scientific) in a reaction volume of 10 µl. The precise conditions for each marker were: 18s Eukaryotic marker 95 ˚C for 10 minutes, followed by 30 cycles of 95 ˚C for 30 seconds, 50 ˚C for 30 seconds and 72 ˚C for 30 seconds, followed by a final extension of 15 mins at 72 ˚C. For the Vertebrate marker, the conditions were 95 ˚C followed by 40 cycles of 95 ˚C for 30 seconds, 48 ˚C for 30 seconds, and 72 ˚C for 30 seconds, followed by a final extension of 15 mins at 72 ˚C. For the invertebrate marker, the conditions were 95 ˚C followed by 45 cycles of 95 ˚C for 30 seconds, 48 ˚C degrees for 30 seconds, and 72 ˚C for 30 seconds followed by a final extension of 15 mins at 72 ˚C. All primers were used at 0.2 µM concentrations and blocking primers were used at a final concentration of 2 µM. After amplification, all samples were checked on 1% agarose gels to ensure successful amplification.

Table 5.1: The markers and blocking primers used in this study

Marker name	Target taxa	Region amplified	Forward sequence	Reverse sequence	Reference
12sV5	Vertebrates	12s	TTAGATACCCCACTATGC	TAGAACAGGCTCCTCTAG	(M De Barba *et al.*, 2014)

16sMAV	Invertebrates	16s	CCAACATCGAGGTCRYAA	ARTTACYNTAGGGATAACAG	(M De Barba *et al.*, 2014)
HomoB	Human blocking	NA	CTATGCTTAGCCCTAAACCTCAACAGTTAAATCAACAAAACTGCT-C3	NA	(M De Barba *et al.*, 2014)
MamMAVB1	Mammals	NA	CCTAGGGATAACAGCGCAATCCTATT-C3	NA	(M De Barba *et al.*, 2014)
Euka-02	Eukaryotes	18s	TGGTGCATGGCCGTTCTTAGT	CATCTAAGGGCATCACAGACC	(Guardiola *et al.*, 2015)

Library preparation and sequencing

The library preparation and sequencing were conducted as outlined in Chapter 2. The samples were run on a Miseq V3 2x300 flow cell for the Eukaryotic samples and a HiSeq mid output 2x150 flowcell for the invertebrate and vertebrate markers.

Data analysis

The sample reads were bioinformatically cleaned and analysed as described in Chapters 2 and 4. Our reference database was constructed from the EMBL ref database (release March 2022) (https://www.ebi.ac.uk/) and the final database was constructed as shown in OBITools3 (Boyer *et al.*, 2016) in the same way in described in Chapter 4. The final reference database used was the same as that described in Chapter 4. Only samples that matched at 90% or higher were kept after assignment; lower than for *L. lutra* due to the less information in the reference database. The data cleaning steps were performed with the metabaR package (Zinger *et al.*, 2021). Contamination was removed form the sample by determining the contaminants present in the, extraction, sequencing and PCR controls sequentially. The samples were then screened for thes contaminants identified and PCRs

where more than 10% of the reads found to be contaminants were removed before downstream analysis. Tag jumps accounted for using tagjumpslayer function in metabaR following the approaches previously suggested in the literature which reduce the abundance of MOTU's relative to their average across the entire dataset (Esling, Lejzerowicz and Pawlowski, 2015) . The remaining steps were carried out as previously described in chapter 4

Statistical analyses were performed in R (version 4.0.3). The coverage of each sample was calculated using Hill numbers in the Metabar package. Site coverage using rarefaction was estimated using the iNEXT package. Diet richness was assessed using the frequency of occurrence (F_O%) (Liu *et al.*, 2020b). Differences in species richness between the sites were tested using Fisher-pitman permutation tests (Coin *et al.*, 2000). We filtered out any family that contained fewer than 50 reads across the entire dataset, based on the limit of detection found from our positive controls (Chapter 2). We calculated relative read abundances to assess differences in diet composition while accounting for the differences in read count between samples with the decosatand function in the vegan package (Dixon, 2003)(Deagle *et al.*, 2019). We calculated Bray-Curtis dissimilarity distances for among sites with the K=3 and 999 iterations with *metaMDS* function (Bray and Curtis, 1957). A perMANOVA (Oksanen, 2008)(Anderson, 2001) was used to investigate if community composition differed between the four sites. We used the adonis2 function in vegan with 999 permutations and tested the data to ensure the assumptions for a perMANOVA were fully met using the betadisper function (*VEGAN, A Package of R Functions for Community Ecology on JSTOR*, no date). Our data were visualised using an NMDS (nonmetric multidimensional scaling) and plotted using ggplot2. The envfit function with 999 permutations was used to identify potential species that could be driving the compositional differences between the samples. Further to this, we investigated if there were any species driving the differences found in the data using the indicspecies package (De Cáceres and Legendre, 2009).

Results

Final dataset description

After bioinformatic and quality filtering, 3,036,239 reads remained of 3,640,984 initial reads over 122 samples, with 16,303 reads per sample on average. For the vertebrate marker, 4,560,974 were identified, and after bioinformatic cleaning 3,443,521 reads remained, with an average number of reads per sample of 11,478. For the invertebrate marker, of an initial 5,014,158 reads, 2,169,468 remained after bioinformatic filtering, with an average number of 25,016 reads per sample. All samples reached full coverage using rarefaction.

Eukaryotic marker

Dietary richness

We found 206.6 unique Eukaryotic MOTU's in each faecal sample on average (SD ± 60.45, range 45–359). The richness found in the individual faecal samples varied between the sites sampled, with the Finca la Sirena being most diverse 219 (SD ± 59.2, range 48–311) with MOTUs being the least diverse with Rio de la passion being having 193.3 MOTUs on average (SD ± 57.6, range 74–322). A full breakdown of diversity of each site is available in (Supplementary Data 5.1 Table S2).

We identified sequences with an 90% match or higher to the reference database. We then subsetted the data to only include metazoan taxa, as other clades are not directly relevant to the diet of *Lontra longicaudis*. After all reads with the same taxonomic identification were merged, we found 340 unique taxa, of which we identified 78% to kingdom, 87% to phylum, 83% to class, 79% to order, 78% to family, 59% to genus and 41% to species level (Supplementary Data 5.1 S1). The Eukaryotic marker identified four relevant Metazoan phyla and the frequency of occurrence in samples was Chordata 94%, Arthropoda 92%, Annelida 7%, and Mollusca 2% (Supplementary Data 5.1 S4); phyla excluded as non-diet relevant included the parasites, Acanthocephala, Platyhelminthes and Nematoda. There were no significant differences between the sites based on the frequency of occurrence of Phyla (Fisher-Pitman Permutation Test, chi-squared = 0.166, p-value = 0.983).

Dietary abundance

Relative rank abundance data showed Chordata to be the most common phyla with 48% of the reads, followed by Arthropoda with 38%, Annelida 11%, and Molluscs 3% (Figure 5.2, Supplementary Data 5.1 S6). There rank order of phlya was consistent between the relative abundance data and the frequency of occurrence. However, proportionately the relative rank abundance of chordates was a little higher relative to Arthropods than it was in the frequency of occurrence data the abundance and frequency of Molluscs and annelids roughly the same. There were no significant differences in the species abundances between sites (perMANOVA df =3, R squared = 0.03 f value = 0.958, P value = 0.529) (Figure 5.3A).

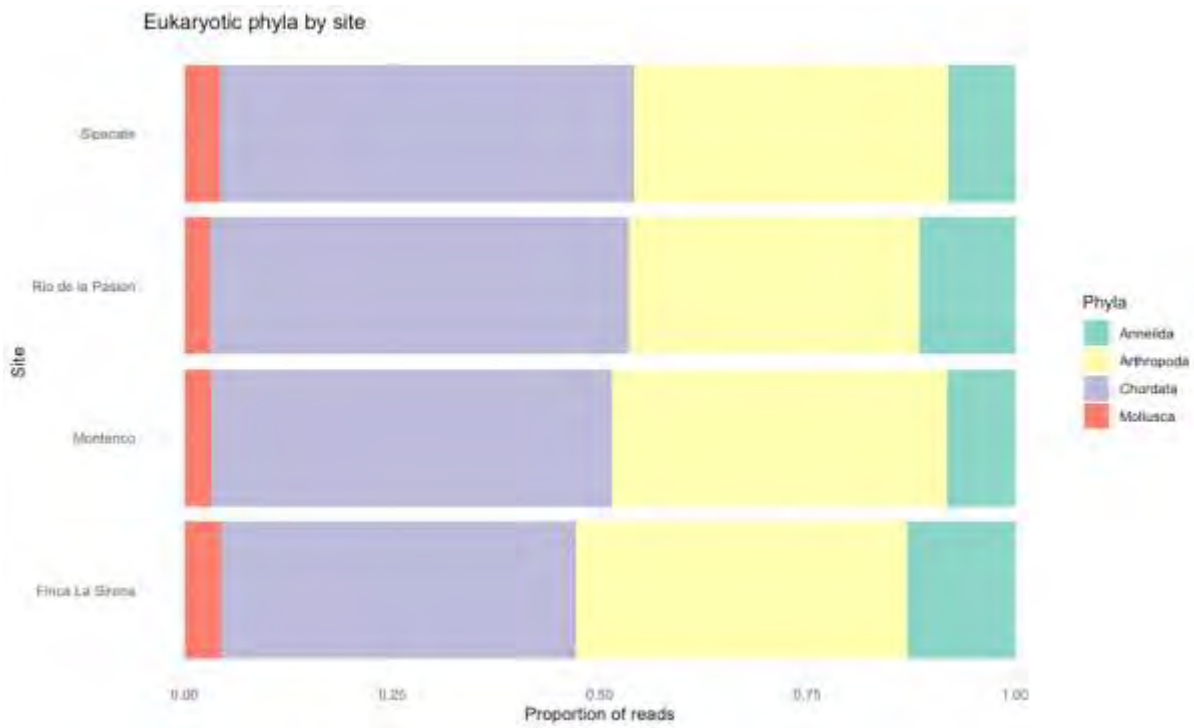

Figure 5.2: A breakdown of the relevant eukaryotic phyla abundances by site. The most abundant phylum across all sites is Chordata with 48% of reads, followed by Arthropoda with 38% of reads with Annelids making a further 13% and Molluscs 3%. There were no significant differences between in the abundances of taxa between sites (perMANOVA df =3, R squared = 0.03 f value = 0.958, P value = 0.529)

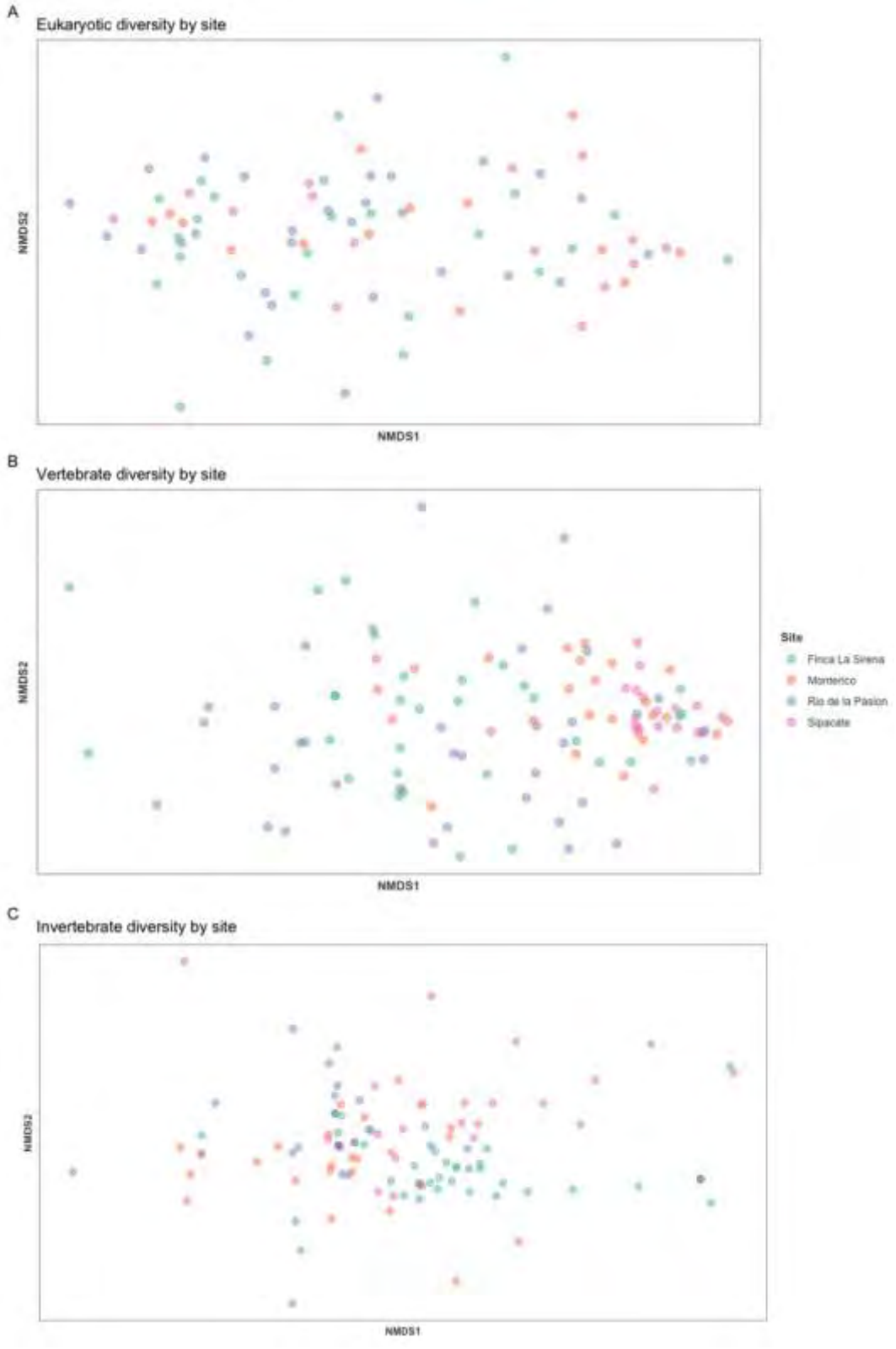

Figure 5.3; NMDS plots of RRA-based Bray-Curtis dissimilarities of Lonra longicaudis diet a) Eukaryotic marker (perMANOVA, df =3, R squared = 0.03 f value = 0.958, P value = 0.529), and b) Vertebrate 12s marker (perMANOVA, 1000 iterations, df = 3, r squared = 0.144, F value = 6.03, p value = <0.001). c) Invertebrate 16s marker (perMANOVA, 1000 iterations, df = 4, r squared = 0.09, F value = 3.65, p value = <0.001),). All stress levels were below 0.2 Clark (1993).

Vertebrate marker

We used human blocking primer to keep contamination to a minimum, and therefore non-target taxa only contributed 13% of our reads. Using the vertebrate marker, we identified 78% of samples to kingdom, 87% to phylum, 79% to order, 83% to class, 78% to family, 60% to genus, and 42% to species level (Supplementary Data 5.2 S1).

We found 31.1 unique vertebrate MOTU's in each faecal sample on average (SD ± 8.97, range 8–51). The richness found in the individual faecal samples varied between the sites sampled, with the Monterico being most diverse 32.7 (SD ± 9.66, range 15–47) with MOTUs being the least diverse with Rio de la passion being having 27.6 MOTUs on average (SD ± 9.44, range 8–48). A full breakdown of diversity of each site is available in (Supplementary Data 5.1 Table S3).

Dietary richness

In total, we found 91 vertebrate taxa that were identified to at least family level. Of the remaining reads, Eleotridae (sleeper gobies) were present in 99% of samples, Loricariidae (armoured catfish) 80%, Cichlidae 67%, Synbranchidae (swamp eel) 67%, Gobiidae (gobies) 47%, Poeciliidae (Livebearers) 39%, Ariidae (catfish) 34%, Suidae (pig) 14%, Odontophoridae (new world quail) 6%, and Phasianidae (turkeys) 2%. All other families occurred in no more than one sample (Supplementary Data 5.2 S4).

On average we found 12 taxa in each sample (SD = ± 9, range = 8-51). Between sites we found no significant differences in species richness (Fisher-Pitman Permutation Test, chi-squared = 6.730, p-value = 0.078). The most diverse site was Monterico (mean richness per sample 32.7, SD ±9.7), while the least diverse site was Sipacate (mean richness per sample 27.7, SD ± 9.44) (Supplementary Data 5.2 S2). Loricariidae (the invasive armoured catfish) was found at all four sites.

Dietary abundance

The relative rank abundance data largely conforms to the richness data. Overall, 81% of the vertebrate reads were from fish families. Eleotridae and Loricariidae were the most abundant families with 19% and 14% of the total number of reads (Figure 5.4).

Synbranchidae and Gobiidae made up a further 11% each, with all other families contributing less than 10% of the total number of reads (Supplementary Data 5.2 S6). Within Eleotridae, the most common species present in our samples were *Eleotris picta* (spotted sleeper goby) and *Gobiomorphus australis* (likely a mis-identification of a related species). Of the Loricariidae family, we mainly found the sub-family of Hypostominae, commonly *Pterygoplichthys etentaculatus*, an invasive species of armoured catfish. Of the Cichlidae family, *Parachromis managuensis* (jaguar cichlid) was the most common cichlid species consumed. While all reads of Synbranchidae were identified *Synbranchus marmoratus*, (marbled eel). No common prey item was a family of commercially important fish. Outside of the four main vertebrate families that made up the diet of *Lontra longicaudis*, the results underlined its opportunistic feeding habits with a wide variety of prey items including pig, caiman, heron, and amphibians.

There were significant differences between sites for relative rank abundances of vertebrates (perMANOVA, 1000 iterations, df = 3, r squared = 0.144, F value = 6.03, p value = <0.001). As expected from geographic location, Monetrico and Sipacate were more similar to each other, and Rio de la Pasion and Finca la Sirena were more similar (Figure 5.3B). There were five species associated with Finca la Sirena, two with Rio de la Pasion, two with Sipacate and one with Monterico (Supplementary Data 5.2 S10).

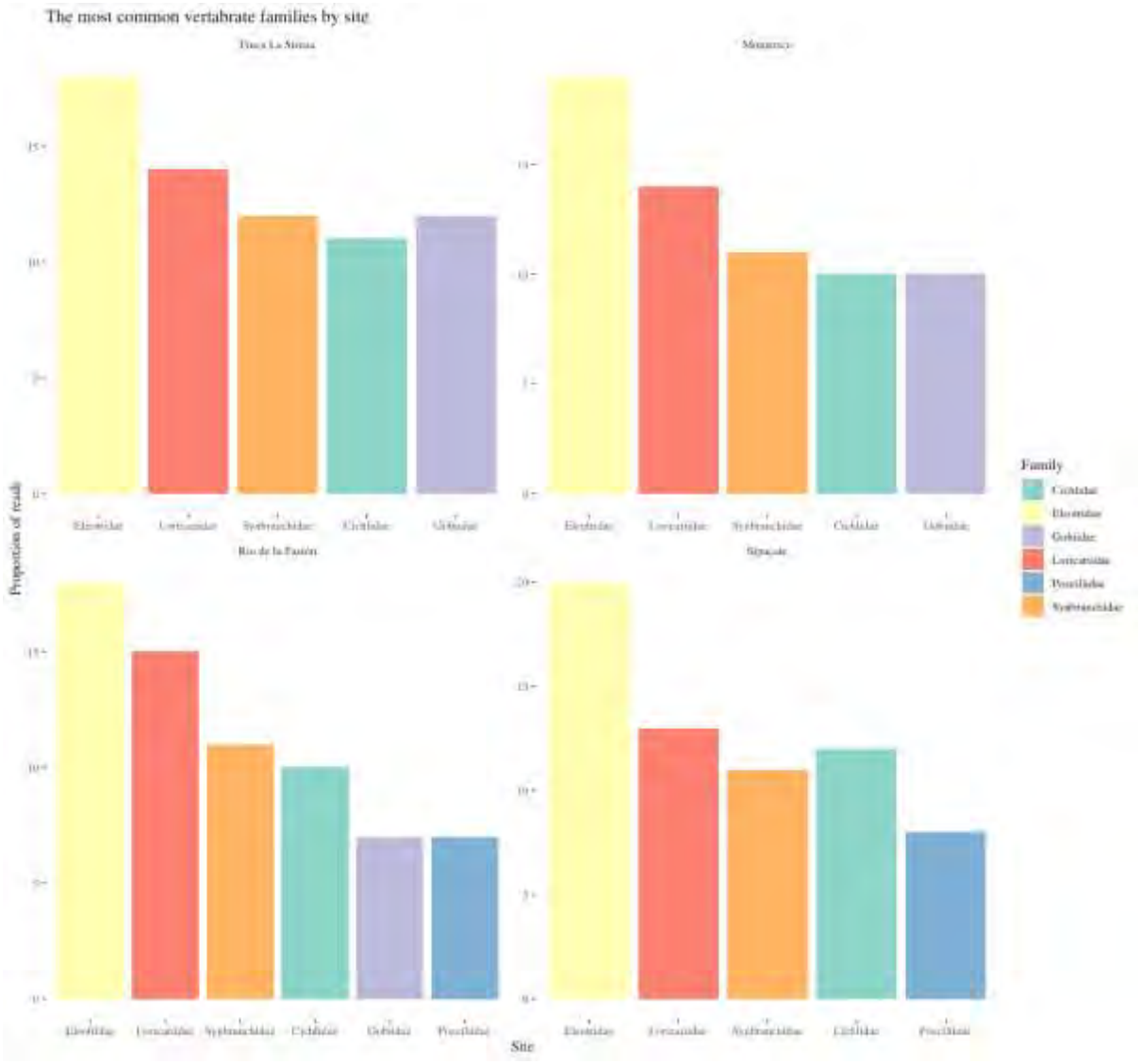

Figure 5.4 A breakdown of the five most common vertebrate families by each site; Eleotridae (sleeper gobies) is the most common family at each site followed by Loricariidae (armoured catfish). These are followed by Synbranchidae (swamp eels), Cichlids, Gobieidae (gobies) and Pociliodae (livebearers).

Invertebrate marker

We used a blocking primer that targeted mammalian taxa to reduce the number of non-target mOTUs in our dataset and this was largely successful. However, we obtained a large number of bacterial mOTUs, which noticeably reduced our read depth. In response, we filtered mOTUs to include only metazoan sequences. In this final dataset, we identified 270 unique taxa, 98.5% to phylum, 86.6% to class, 72.5% to order, 52.5% to family, 41.5% to genus, and 30.5% to species level (Supplementary Data 5.3 S1).

Dietary richness

We found 32.7 unique invertebrate MOTU's in each faecal sample on average (SD ± 12.42, range 7–72). The richness found in the individual faecal samples varied between the sites sampled, with the Finca la Sirena being most diverse 35.2 (SD ± 12.09, range 16–72) with MOTUs being the least diverse with Rio de la passion being having 28.05 MOTUs on average (SD ± 9.83, range 15–58). A full breakdown of diversity of each site is available in (Supplementary Data 5.1 Table S3).

In total, we identified 93 families, but only 26 of these occurred in ≥1% of samples and nine in ≥5% of samples. We excluded six families as not relevant to otter diet, including Formicidae (ants), Dicyrtomidae (springtails) and Chrysomelidae (leaf beetles). The most frequently occurring family was Portunidae (crab) occurring in 38% of samples. The next most common diet-relevant family was Palaemonidae (shrimp) occurring in 22% of samples and Pseudothelphusidae (Crab) in 14% of samples. Of the Portunidae family, the most common species identification was *Callinectes sapidus* (blue crab) followed by *Callinectes exasperates* (Rugose swimming crab). In the Palaemonidae family, the most commonly occurring species were *Macrobrachium shokitai* and *Macrobrachium rosenbergii* (the giant freshwater prawn) All mOTUs assigned to the Pseudothelphusidae f*amily*, *were Potamocarcinus chajulensis*.

There were differences in invertebrate species composition between sites (F*isher-Pitman Permutation Test*, chi-squared = 25.511, P < 0.001). Finca La Sirena had, with Palaemonidae shrimp occurring in 61% of samples compared to (Supplementary Data 5.3 S3) 15% of samples at Sipacate, 14% of samples at Monterico and 3% at Rio de la Pasion. Portunidae were ubiquitous occurring in 50% of samples at Sipacate, 40% at Finca la Sirena, 31% at Monterico and 28% at Rio de la Pasion. Pseudothelphusidae occurred in 22% of Rio de la Pasion, 20% at Sipacate, 14% at Monterico and 6% at Finca la Sirena.

Dietary abundance

The relative read abundance data showed a different picture to the dietary richness data. The most abundant family was Portunidae (crab) with 13% of the reads. Palaemonidae,

which was the most frequently occurring family, represented 10% of the reads. Pseudothelphusidae represented 6% of the reads. *Callinectes sapidus* (blue crab) was the most abundant species identified for Portunidae, whilst *Macrobrachium shokitai* was the most abundant for Palaemonidae, closely followed by *Macrobrachium rosenbergii*. The relative abundances of the invertebrate families at the four sites are shown in Figure 5.5.

There were significant differences in relative rank abundances amongst the sites (perMANOVA, 1000 iterations, df = 4, r squared = 0.09, F value = 3.65, p value = <0.001), although the difference are not large (Figure 5.3C). Finca la Sirena is more distinct than other sites on the axis 1; other sites show greater dispersion on axis 2 (Figure 5.3C). We found 31 taxa associated with one site, one taxa associated with two sites and one taxa associated with three sites. *Callinectes sapidus* (Blue crab) and *Macrobrachium rosenbergii* (giant freshwater shrimp) were significantly associated with Finca la Sirena and not found elsewhere (Supplementary Data 5.3 S9).

Overall diet

Overall from the Eukaryote marker, we found that, once non-metazoans were removed, 48% of abundance was vertebrates and 52% was invertebrate (arthropod, annelid and mollusc) reads. We consider that 100% of the vertebrate taxa were relevant to the diet, but only 29% of the invertebrate. This means that invertebrates make up 15% of the diet in terms of abundance. This is likely to be an underestimate because we know that this invertebrate marker has been less reliable when giving relative abundances (Chapter 2).

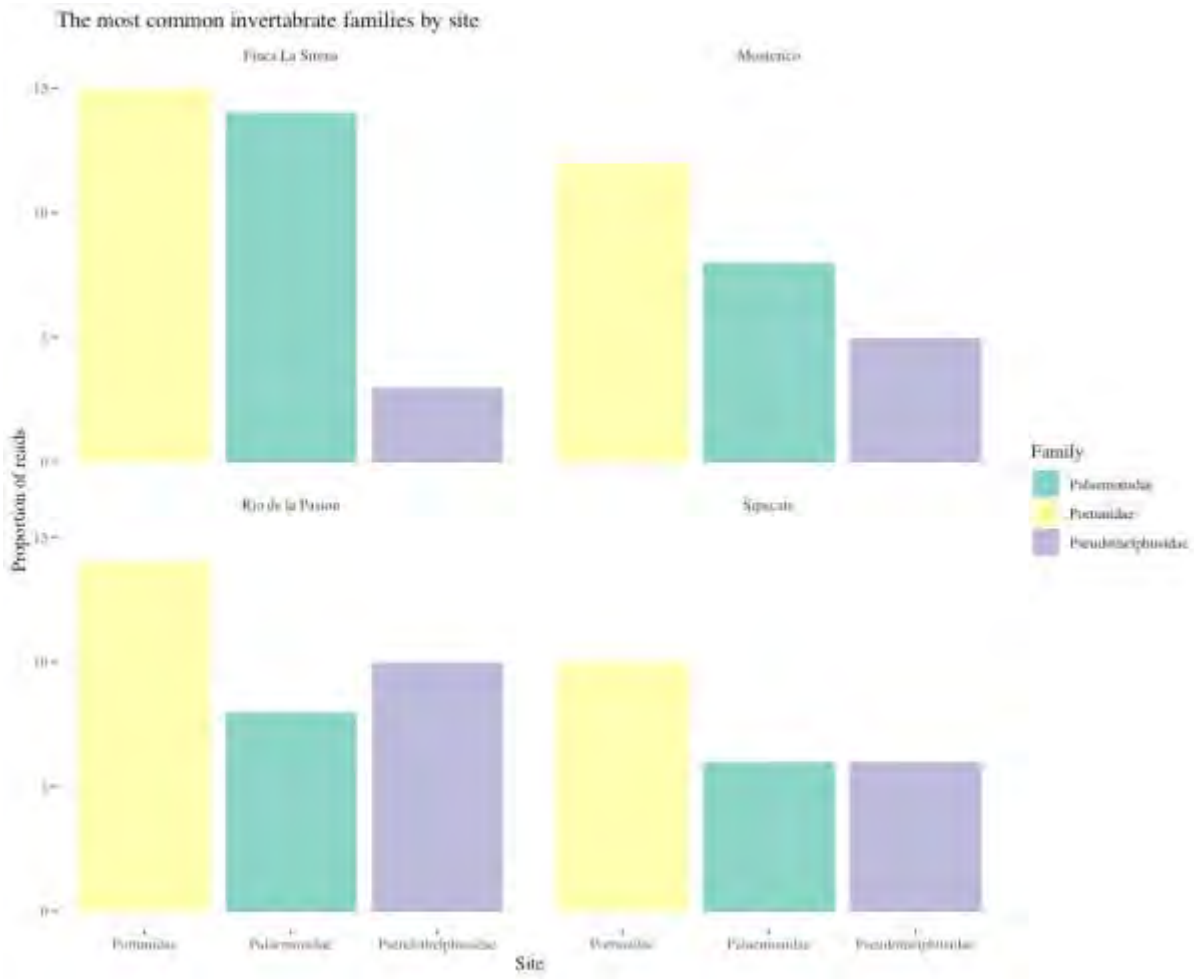

Figure 5.5: Most common diet-relevant invertebrate families by each site; Portunidae (crab), Paaemonidae (shrimp) and Pseudothelphusidae (crab)

Discussion

Information on diet is critically important in the conservation of *Lontra longicaudis* (de Almeida and Ramos Pereira, 2017b; Rheingantz, Santiago-Plata and Trinca, 2017). Our results show the full breadth of the diet consumed by *L. longicaudis* across several sites in Guatemala, Central America. We identified 94 families in our faecal samples that we considered relevant to otter diet. We then used more specific markers to identify components of the diet with more detailed taxonomic resolution. In total, we found 91 vertebrate and 3 invertebrate families. Although the presence of invertebrates in *L. longicaudis* diet was known before this study, the relative importance within the diet was

not known in Central American populations (Juarez-Sanchez, Blake and Hellgren, 2019). We found that invertebrates comprised 15% of the otter diet, higher than found in the Eurasian Otter, *Lutra lutra* (Chapter 4).

We identified 72% of the vertebrates and 58% of the invertebrate mOTUs to at least family level. In the case of vertebrates, we found 53 more taxa in our samples than a recent morphological study in Guatemala, which used more than twice the number of samples included in our study (Juarez-Sanchez, Blake and Hellgren, 2019). Other previous morphological studies have identified only 15 to 37 taxa despite having much larger sample sizes than our study (Quadros and Monteiro-Filho, 1999; Vezzosi *et al.*, 2014; Juarez-Sanchez, Blake and Hellgren, 2019). Moreover, we were able to identify far more taxa to more detailed taxonomic groups, especially non-fish groups that previous studies were unable to identify past general reptilian and mammalian groups (Quadros and Monteiro-Filho, 1999; Mayor-Victoria and Botero-Botero, 2010; Vezzosi *et al.*, 2014) (Silva, Nascimento and Quintela, 2012; Sousa, Bastazini and Colares, 2013). However, the nature of the invertebrate marker, in particular, means it was sometimes difficult to distinguish between prey items and incidental consumption (i.e. prey of prey) (POMPANON *et al.*, 2012). For example, ants were excluded from the dataset but occurred in many samples. Ants are extremely unlikely to be otter prey items but are more likely to represent later contamination by ants feeding on the spraint. Some arthropods, such as crabs and shrimp are likely preyed upon by otters, and we have behavioural studies to support this, but it is also likely that a percentage of the reads arise from fish prey that ate crabs and shrimp.

Overall, 81% of the vertebrate portion of the diet was fish, which was a consistent pattern across all sites. Sleeper gobies were the most abundant taxa in the diet in all sites. The second most common fish family was armoured catfishes. However, beyond these two fish families, we found the sites investigated in this study had large differences in both species composition and abundance in the otter diet, particularly in the invertebrate portion of the diet. For example, *Callinectes sapidus* (Blue crab) and *Macrobrachium rosenbergii* (giant freshwater shrimp) were dominant on the Caribbean coast, and *Potamocarcinus chajulensis* was dominant on the Pacific coast. This was expected as there are widely understood biogeographic patterns in insects, amphibians, and freshwater fish in Central America (Bagley and Johnson, 2014). Fish diversity, for

example, is known to be highly endemic in Central America, with large differences between the Atlantic and Pacific coasts (Briggs, 1984).

We identified invasive catfish in the otter diet in all four sites we collected samples from. These invasive species were present in high abundance at all locations. This confirms what has been previously recorded in other otter diet studies, as well as other surveys looking at northern rivers such as Rio de la Passion and San Pedro (Juarez-Sanchez, Blake and Hellgren, 2019). However, we also have found armoured catfish in more southerly river systems, such as Rio Dulce and Sipacate, where currently it is under-reported, or not reported at all as far as we are aware. The spread of this species is worrying due to its implications for native fish species' declines (Gaitán *et al.*, 2020). This demonstrates the efficacy of metabarcoding diet studies for identifying the presence of invasive species by monitoring the diet of a generalist piscivore.

Our study reinforces the idea that metabarcoding studies can accelerate and improve the quality of information generated by diet studies (Kartzinel *et al.*, 2015; Ruppert, Kline and Rahman, 2019). However, our study is also limited by many of the same things that have limited previous metabarcoding projects. Identification rates to species level in the invertebrate marker were 42%, far lower than would be ideal due to incomplete reference databases. This problem is especially acute in tropical and subtropical regions with their high levels of diversity and relatively understudied fauna compared to more temperate zones. Future efforts to recreate more comprehensive reference databases are, therefore, essential if high throughput studies are to reach their potential (Coissac, Riaz and Puillandre, 2012). Furthermore, the incomplete nature of the reference databases and markers with less than 100% resolution make misidentifications likely (Piper *et al.*, 2019). For some species, this misidentification could be found, e.g. for species not present in the Americas, and the species removed from the analysis, but this was not possible for an unknown number of species that remained in the database. Where misidentified species were removed, this would also have resulted in a false negative, with the removal of a species that was a true part of the diet. For two of our markers, we struggled to get good amplification. The main reason for this was the length of time between collection and DNA extraction (Alberdi *et al.*, 2018). Our samples were kept in

sub-optimal conditions because of the coronavirus outbreak, and this likely led to some degradation, making amplification of the samples more difficult (Massey *et al.*, 2021).

Pig and cow were the commonest mammals in the faecal samples. It is very unlikely that *Lontra longicaudis* is actively hunting these species. It may be evidence of scavenging behaviour or it may be that their prey items have scavenged. Of birds, the most common species identified were chicken, green heron, and several parrot species. Despite identifying these taxa, they remain a small proportion of the total diet, 10% in total.

Here we present the first metabarcoding study on *Lontra longicaudis* diet, and we show that by using hierarchical markers, we can study a broad range of prey items with a high level of identification, far higher than traditional morphological studies have managed in the past. We were also able to determine the relative contributions of invertebrates and vertebrates to the diet, highlighting the important role invertebrates play in otter diet. Morphological studies are usually limited to small regions or one river basin (Quadros and Monteiro-Filho, 1999; Mayor-Victoria and Botero-Botero, 2010). However, our approach allowed us to investigate four sites across three separate regions of Guatemala, using a limited number of samples. This highlights the ability of metabarcoding studies to survey over much greater scales, more quickly and accurately than would previously have been possible (Taberlet *et al.*, 2018). Although metabarcoding studies are not cheap, the insights provided are extremely powerful, and the possibility to work at scale brings down the cost per sample to levels far below traditional studies, meaning it is a cost-effective and more accurate way of accumulating information crucial to species conservation (Nichols, Åkesson and Kjellander, 2016). We also found the presence of an invasive species of armoured catfish at all sites, demonstrating both their range expansion and the potential for diet studies to monitor changes in ecosystems.

Data availability,

All supplementary information files are available in a public repository on Zenodo.com https://doi.org/10.5281/zenodo.8060380 raw sequencing files will be available upon publication.

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Chapter 6 – Investigation into the diet of the critically endangered Bermuda Skink, *Plestiodon longirostris*

Abstract

In order to effectively conserve a species both in-situ and ex-situ, accurate information is needed on its habitat, behaviour and habits in the wild, including its diet. The Bermuda skink, *Plestiodon longirostris*, is a critically endangered lizard that has undergone population decline in recent centuries. It is the subject of in-situ and ex-situ conservation actions, yet we know little of its dietary needs and even less about its natural diet. The small and isolated populations of this species have made diet studies difficult to carry out, and only a few studies have been conducted. Those that are available have low taxonomic resolution resulting in a paucity of information that is impeding both conservation efforts for this species. Here, we use eDNA gained from faecal samples to uncover the diet of the Bermuda skink. These samples (n=98) were collected from three wild skink populations on three islands in Bermuda (Nonsuch, Castle, and Southampton Islands). The resultys indicate plants, invertebrates and vertebrates contributed equally to the diet. This was consistent between the sites, although the taxa within those groups varied. We find substantial evidence of scavenging behaviour, both from birds and humans. The skink diet reflected the diverse nature of the islands, with Nonsuch Island, a nature reserve, having more native plants in the diet. Overall, we demonstrate that the skink is an opportunistic scavenger and generalist omnivore, which was previously unknown and is information that will aid ex-situ and in-situ conservation.

Introduction

Earth is currently approaching another mass extinction event driven by anthropomorphic actions that have caused habitat degradation and fragmentation as well as the introduction of invasive species further accelerating species decline (Gurevitch and

Padilla, 2004; Vitousek *et al.*, 2008). In order to slow or reverse biodiversity loss, it is critical to obtain relevant ecological data that allow us to formulate effective conservation strategies (de Sousa, Silva and Xavier, 2019). Understanding a species' diet is essential for both in-situ conservation efforts, allowing us to prioritise habitat management (Antwis and Browne, 2009; Williams *et al.*, 2022)and ex-situ conservation, by providing effective nutrition to captive individuals (Ogilvy, Preziosi and Fidgett, 2012). Providing a realistic diet can also help captive individuals develop the skills needed for ex situ bread individuals to survive when released into the wild (Sanders and Fernandez, 2020).

Metabarcoding has increasingly shown itself to be an effective tool for investigating diet and has been utilised on a wide variety of species, including carnivores and herbivores (Kartzinel *et al.*, 2015; de Vos *et al.*, 2018; Shi *et al.*, 2021). Metabarcoding studies can identify prey items with a higher taxonomic resolution and on a larger scale, both geographically and in terms of sample size, than traditional diet studies (Massey *et al.*, 2021; Pertoldi *et al.*, 2021). Metabarcoding gives the added advantage that it is not reliant on highly skilled taxonomists to identify partially digested prey items and is less susceptible to error, provided there is a sufficiently representative genetic database available to identify the items (Ruppert, Kline and Rahman, 2019). Metabarcoding studies also have the advantage of being able to identify prey items that are mostly digested and no longer have any structures that are easily morphologically identifiable, which would lead to them being missed by a traditional analysis (Carss and Elston, 1996; Carss and Parkinson, 1996). The speed at which metabarcoding can be used to generate data at a population scale makes it an ideal choice for generating data from species that are difficult to sample due to their elusive nature or small stature (Piper *et al.*, 2019).

One of the groups that is most impacted by changes in habitat and the effects of climate change are reptiles, with a recent study finding reptiles to be undergoing some of the most serious declines of all phyla outside of amphibians (Cox *et al.*, 2022)Therefore, increasing our knowledge of what reptilian species need to survive is a high priority for conversation efforts. The Bermuda skink, *Plestiodon longirostris,* is a critically endangered lizard that is endemic to Bermuda and its surrounding islands (Coughlan *et al.*, 2004) and the only terrestrial vertebrate that is native to Bermuda. It has undergone large population declines due to habitat loss, pollution and invasive species (Davenport *et al.*, 2001). Currently, we believe the population to be extremely vulnerable to a potential

extinction with a single storm event capable of wiping out the whole population (Davenport *et al.*, 2001)Accordingly, recent efforts have been made by Chester Zoo to establish an ex-situ population with the aim of ensuring their survival (Turner, 2019). However, the success of any conservation program, either ex situ or in situ, is dependent on determining the relevant ecological conditions the Bermuda skink needs to survive (Dolman *et al.*, 2015). Yet, despite its cultural and ecological importance to the islands of Bermuda, we still know relatively little about its ecology (Turner, 2018). Currently almost nothing is known about the Bermudan skink. Some studies have suggested a diet based on that of other lizards in the region (e.g. Stroud *et al.,* 2017) and others have proposed preferences based on feeding preference experiments of ex-situ populations, with the latter finding fruit-based diets are more preferred (Williams *et al.*, 2022).

In this study, we use a DNA metabarcoding approach to explore the diet of the Bermuda skink and to identify the most important constituents. We also investigate variation in the diet within and between islands. We believe that this information will be crucial in helping the captive breeding programme and subsequent potential reintroductions.

Methods

All data was collected by Chester zoo in 2018 with samples collected under a licence overseen by Gerardo Garcia. The sequencing undertaken here is part of the larger conservation project led by Chester Zoo.

Study species

The current status of the Bermuda skink has been established through a small number of population surveys that have been conducted sporadically throughout the 20th and 21st centuries (Coughlan *et al.*, 2004; Turner, 2018). Bermuda skinks have been described as uncommon since the early 20th century and are largely restricted to several islands separate from the main island (David B. Wingate, 1965). The current population structure was first described in the 1990s as a mixture of a classic metapopulation and a main island model. Such a structure leaves the whole species vulnerable to extinction because if the main population is lost, the sub-populations may not have the genetic capacity to survive alone (Turner, 2018). The most recent work undertaken looked at using PIT tags to determine the daily routine of Bermuda skinks across the various

locations, looking at the demography and health of individuals in those populations. That work is the first demonstration that human disturbance negatively affects those populations (Turner, 2019). An in-depth look at the diet of these skinks provides complementary information that will help conserve this critically endangered lizard.

Sample collection

Ninety-eight Bermuda Skink faecal samples were collected from the Nonsuch, Castle and Southampton Islands in Bermuda in 2017 by H. S. Turner (Turner, 2018) (Figure 6.1). Skinks were attracted to baited jar traps using cheese and sardines, and the skinks were kept in jars until they defecated. Faeces were then collected, and the skinks were released (Turner, 2018). Samples were stored in NAP buffer (Camacho-Sanchez *et al.*, 2013) at room temperature and then transported to the UK where DNA extraction was carried out at Manchester Metropolitan University. Seven samples were collected from Nonsuch, 33 from Castle and 45 from Southampton Island and an additional 13 samples for which the island of origin is unknown.

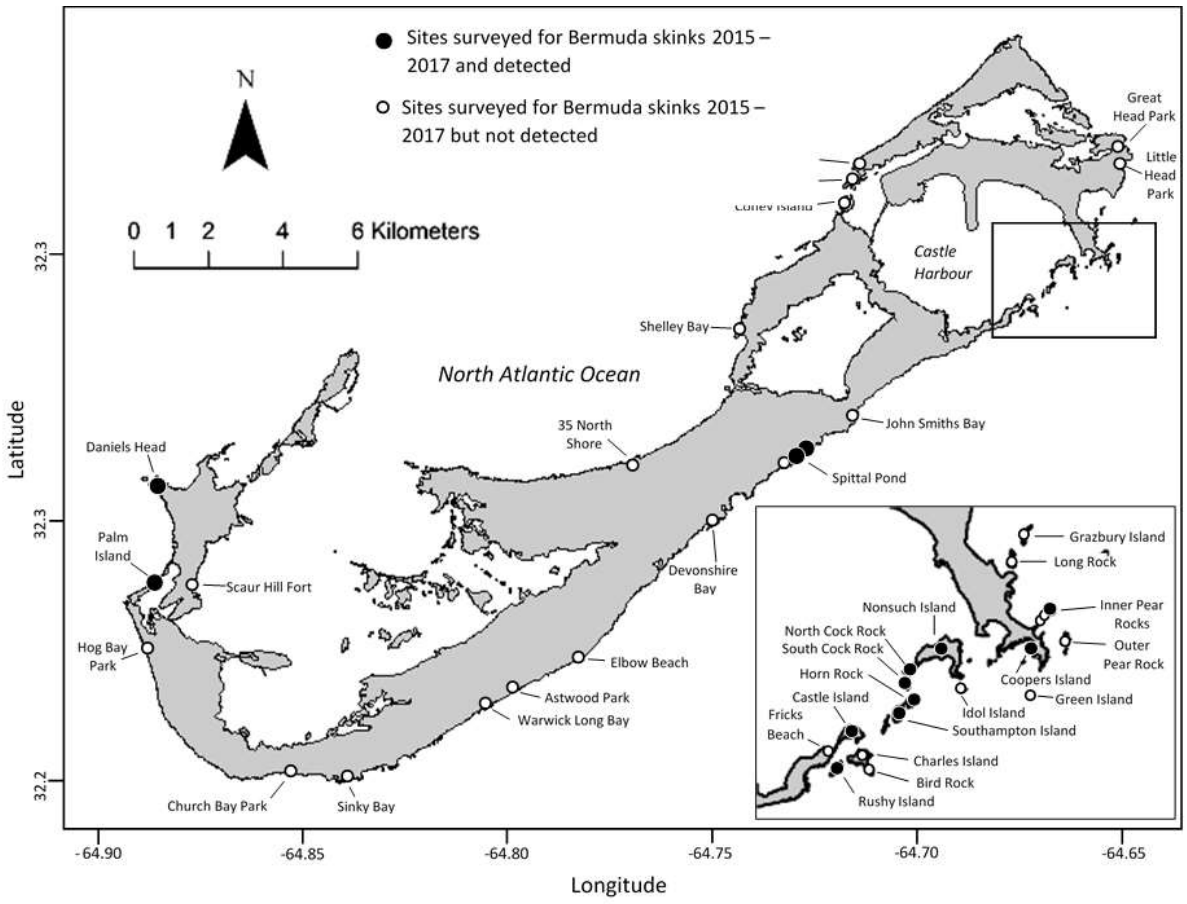

Figure 6.1 Sites sampled by Chester Zoo and the University of Kent for the presence and absence of Bermuda Skink. The faecal samples analysed here were collected from Southampton, Nonsuch, and Castle Islands; the other surveyed islands did not provide any faecal material. Figure taken from Turner (2018)

DNA extraction

The DNA was extracted from faecal samples using the same methodology as presented in Chapter 4. A total of 98 samples were taken forward for analysis, normalised and put into a randomised stratified plate design as described in Chapter 4, following Taberlet *et al*. (2018).

Marker Choice and PCR conditions

The marker used for analysing the diet was 18s Eukaryotic marker (Taberlet *et al.*, 2018). We chose a higher taxonomic marker (Table 6.1) because of the suspected generalist feeding habits of the Bermuda skink. The optimum cycle number and annealing temperature were determined as described in Chapter 4. The final PCRs were amplified with Amplitaq gold 360 master mix (Thermo Fisher Scientific), in a reaction volume of 10 µl. The precise conditions for each marker were: 18s Eukaryotic marker - 95 ˚C degrees followed by 30 cycles of 95 ˚C for 30 seconds, 50 ˚C for 30 seconds and 72 ˚C for 30 seconds, followed by a final extension of 15 mins at 72 ˚C. The primers were used at 0.4 µM concentrations. After amplification, all samples were checked on 1% agarose gels to ensure successful amplification. All samples were present in the final pool even if no visible band was present on the gel.

Table 6.1: The marker used in this study

Marker name	Target taxa	Region amplified	Forward sequence	Reverse sequence	Reference
Euka-02	Eukaryotes	18s	TGGTGCATGGCCGTTCTTAGT	CATCTAAGGGCATCACAGACC	(Taberlet *et al.*, 2018)

Library preparation and sequencing

The library preparation and sequencing were conducted as outlined in Chapter 2. The samples were run on a Miseq V2 2x250bp nano flow cell.

Data analysis

The sample reads were bioinformatically cleaned and analysed as described in Chapters 2 and 4. Our reference database was constructed from the EMBL ref database (release March 2022) (https://www.ebi.ac.uk/) and the final database was constructed as shown in OBITools3 (Boyer *et al.*, 2016) in the same way in described in Chapter 4. The final reference database used was the same as that described in Chapter 4. Only samples that matched at 95% or higher were kept after assignment. In the case of chordates, the low number of species in our reference database meant that the most common sequences were identified individually with NCBI Blast and any misidentified reads were removed prior to analysis. The sequences that were removed and their EMBL and NCBI identifications are present in the Supplementary Data 6.1. The resulting taxonomically assigned sequences were then imported into R version 4.0.3 The data cleaning steps were perfomed with the metabaR package (Zinger *et al.*, 2021). Contamination was removed form the sample by determining the contaminants present in the, extraction, sequencing and PCR controls individually. The samples were screened for recognised contaminants where more than 10% of the reads found in a sample were contaminants the sample was removed before further downstream analysis. Tag jumps accounted for using tagjumpslayer function in metabaR following previous approaches (Esling, Lejzerowicz and Pawlowski, 2015) MOTU's are reduced across the entire dataset relative to their ambiences in the sequencing controls thereby negating the impact of tag jump. The remaining data cleaning steps were carried out as previously described in chapter 4. Finally, the replicates were then bioinformatically pooled into individual samples, and the resulting composite samples were analysed for ecological differences. Sequences that were identified only to higher taxonomic ranks, such as Phylum or Order were only used in analyses where those ranks were relevant.

Diet richness was assessed using the frequency of occurrence (F_O%) (Liu *et al.*, 2020b). Differences in species richness between the sites were tested using Fisher-pitman

permutation tests (Coin *et al.*, 2000). We calculated relative read abundances to assess differences in diet composition while accounting for the differences in read count between samples with the decosatand function in the vegan package (Dixon, 2003)(Deagle *et al.*, 2019). We calculated Bray-Curtis dissimilarity distances with respect to the different sites with the K=2 and 999 iterations with *metaMDS* function (Bray and Curtis, 1957). A PERMANOVA (Oksanen, 2008)(Anderson, 2001) was used to investigate the effects of island. We used the adonis2 function in vegan with 999 permutations and tested the data to ensure the assumptions for a PERMANOVA were fully met using the betadisper function (Oksanen, 2008). Our data was visualised using an NMDS (nonmetric multidimensional scaling), and plotted using ggplot2 (Villanueva and Chen, 2019) using themes from ggthemes (Arnold and Arnold, 2015). We investigated if there were any species driving the differences found in the data using the indicspecies package (De Cáceres and Legendre, 2009).

Results

Final data set description

Of the initial total of 98 initial samples, 30 were removed from further analysis, 28 of which were removed for poor amplification as they did not have sufficient read counts, and two samples were removed as they failed the bioinformatic filtering parameters. The remaining samples comprised 26 from Castle Island, seven from Nonsuch Island and 26 from Southampton Island, plus an additional 9 samples whose origin is unknown. From the remaining 68 samples, we identified 13,992 mOTUs belonging to 542 taxa. After bioinformatic filtering and taxonomic identification 2,551,784 reads were left in the database. Sequences that belonged outside the Super Kingdom of Eukaryota were removed. Of the remaining sequences, we identified 89.8% to Kingdom level, 97.7% to Phylum, 83.4% to Class, 89.1% to Order, 72.0% to Family, 35.7% to Genus and 24.2% to Species level. For our analysis, we further partitioned the dataset into metazoan and Viridiplantae sequences, as these are the clades that are relevant to the diet of the Bermuda skink. The number of reads identified as Viridiplantae were 18,421, of which Streptophyta represented the vast majority with 17,883 reads. Metazoan reads totalled 719,656.

Of the 542 taxa, 199 taxa were metazoan, with 109 families identified, 106 genera and 98 species identified. There were 55 taxa identified in the Streptophyta subset, with 27 families, 20 genera, and 14 species. For streptophytans, 100% were identified to class, 89.4% to order, 87.3% to family, 48.8% to genus and 45.1% to species levels. For metazoans, we identified 99.7% to phylum level, 86.6% to class, 97.3% to order, 70.5% to family, 20.9% to genus, and 15.1% to species level.

We considered only chordates, arthropods and molluscs to be the only Metazoan taxa likely to be consumed by skink. Other taxa not considered relevant to skink diet were removed. These included a wide range of organisms, e.g. nematodes, platyhelminths, rotifers and Evosea (amoeba). Nematodes appeared in 44% of our samples, although these could have either been ingested accidentally or could be parasites of the skinks. However, it was not possible to determine which Streptophyta might realistically be eaten. Further to this MOTU's are identified only to family due to the broad nature of the Eukaryotic marker we found ide identification to genus and species level to be spurious.

The identifications of each MOTU to species level were they were obtained are provided in the supplementary information.

Overall, we found 12% of relevant eurkaryotic reads were Chordate, 12% Streptophyta, 11% Arthropoa and 1% Mollusca. We can therefore suggest that across the whole diet 33% comprises Chordate, 33% Streptophyta, and 33% invertebrate.

Dietary richness

Of the relevant phyla to the skink diet, we identified the most frequent were chordates present in 100% of samples, followed by Streptophyta present in 95% of samples, Arthropoda present in 86% of samples, and molluscs present in 9% of the samples. All other potential prey taxa were present in less than 5% of the samples.

Chordata were found in 98% of skink faecal samples. The most common Chordate class was Aves at 78% of samples. The sequence identified as belonging to birds was a 100% match for many avian species; therefore, we do not present the species level identification. Insect was the second most frequent class (43%), and Flatidae (plant hopper) and Formicidae (ants) were the most Insect families. Actintopteri were common (Figure 6.2A), with Labridae (wrasse) as the most common family within Actintopteri. Chondrichthyes were also common, which were mostly identified as Elasmobranchs, found in 10% of samples. Other Classes and Families were present but at less than 10% or less. The most common metazoan classes were mostly the same between islands (Figure 6.2B). Malacostraca (spider crab) were only common on Nonsuch Island.

Of the Streptoptophytan sequences that were present in 95% of samples, the most common families were Asteraceae (in 30% of samples) Poaceae (27%), Polygonaceae (25%), Fabaceae (23%) and Brassicaceae and Casuarinaceae (Sheoaks) both at 17% (Figure 6.3A). All other families were present in less than 10% of our samples. The most common Stryptophyan families differed between the islands, with Fabaceae being common on Nonsuch and Southampton Islands, but Casuarinaceae being only dominant on Castle Island (Figure 6.3B).

Figure 6.2: Frequency of occurrence of the six most common metazoan classes for A) the overall dataset, and B) for each island separately.

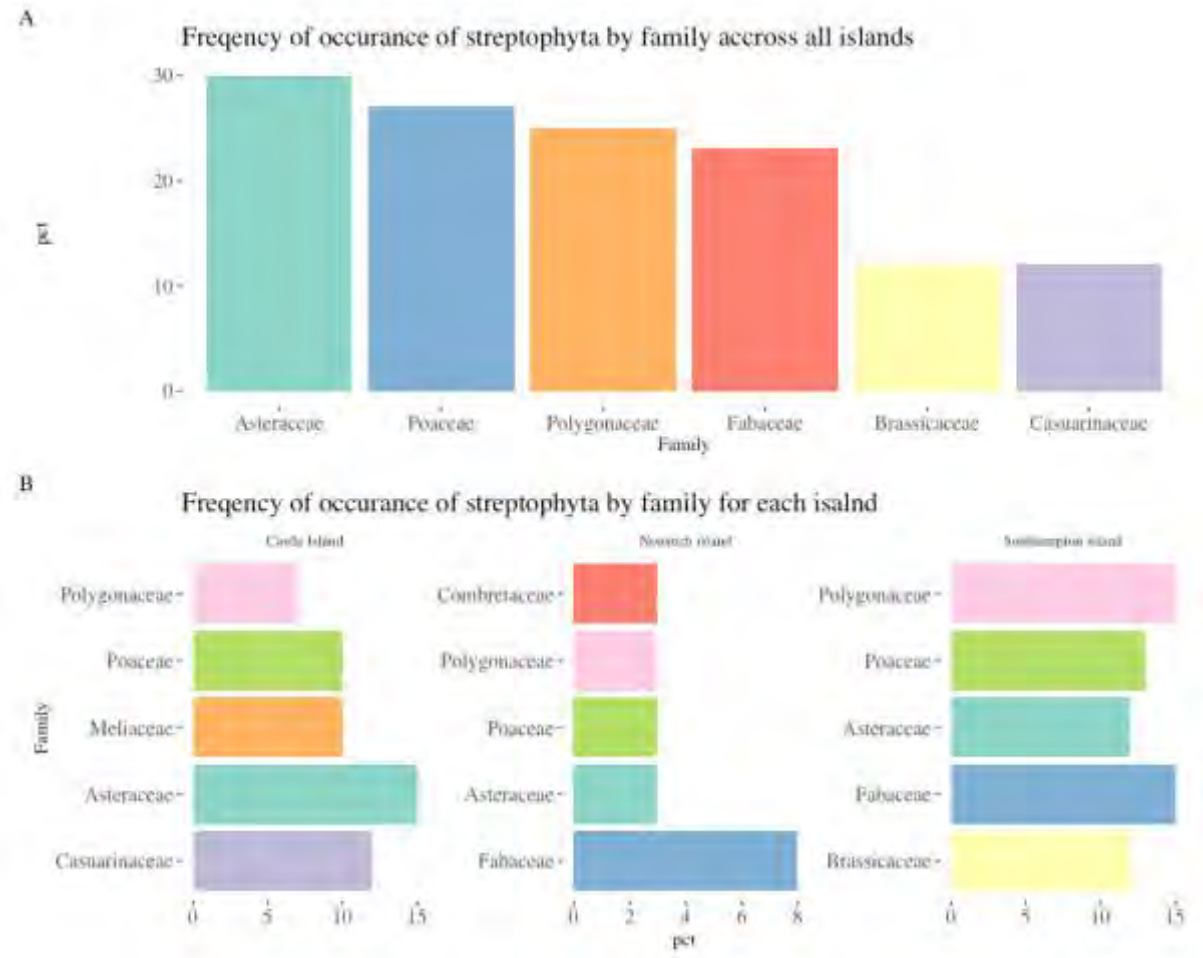

Figure 6.3: Frequency of occurrence of the most common Streptophyan orders for A) the overall dataset, and B) for each island separately.

On average, we found 56 unique Eukaryotic MOTU's in each faecal sample (SD ± 26, range 14–125). The richness found in the individual faecal samples varied between the islands sampled, with Castle Island being the least diverse with 45 taxa on average (Table 6.2). There was no significant difference in the metazoan species richness between the islands (Fisher-Pitman permuatation test, chi-squared = 2.4152 p-value = 0.3084). There was an average of 5.7 (SD ± 3.4, range: 1–17) Streptophyta taxa across all samples, and the variation between islands was small (Table 6.2).

Table 6.2: The number of taxa present in the skink samples for all taxa, metazoans and streptophytans by island. Metazoan were filtered to only include diet relevant taxa (chordates, arthropods and molluscs). Castle Island n = 26; Nonsuch Island n = 7; Southampton Island n = 26.

Numbers of taxa identified in samples				
Site	**Min**	**Mean**	**Max**	**SD**
Castle Island	19	45.2	86	18.9
Nonsuch Island	44	72.1	125	32.0
Southampton Island	14	52.8	110	26.3
Metazoa taxa				
Site	**Min**	**Mean**	**Max**	**SD**
Castle Island	5	10.8	27	4.9
Nonsuch Island	9	18.0	35	11.4
Southampton Island	4	13.5	33	8.1
Streptophyta				
Site	**Min**	**Mean**	**Max**	**SD**
Castle Island	1	5.5	16	3.6
Nonsuch Island	4	6.4	9	2.0
Southampton Island	1	5.3	15	3.6

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Diet composition

Diet composition was assessed with relative read abundances. Streptophyta and Chordata were the joint most abundant taxa with 12% of total reads. Arthropodea was found to constitute 11% of total reads. All other taxa made up less than 5% of the data but included Echinodermata and Mollusca with 3 and 1% of reads, respectively. Other phyla were considered incidental such as fungi, algae, rotifers, and corals, as well as potentially parasitic taxa such as nematodes (Figure 6.4A). There were significant differences between islands with Southampton being less dispersed than the other two islands (Figure 5A, perMANOVA, r-squared = 0.068, F = 2.069, p-value < 0.001).

There were significant differences in the diet relevant metazoan abundances between the islands (perMANOVA, r-squared = 0.05, F = 1.73, p-value = 0.006), with Southampton Island being much less dispersed than the other islands (Figure 6.5B). Within Chordata, while the most abundant taxa is Aves at 50%. The remaining taxa all make up substantially lower abundances with Muridae (rats) making up 11%, Odontaspididae (sand sharks) accounted for 7%, Labridae (wrasses) made up 5%. All other families were present at negligible levels. Of the Arthropods reads, the most abundant families were Formicidae. There were clear differences in the arthropod abundances between Nonsuch Island and the other islands. Most abundant read on Nonsuch Island was Monoplistidae (scaly cricket), whereas Formicidae (ants) were common on the other two islands (Figure 6.4D).

Of the Streptohytan reads, the most abundant families were Polygonaceae (buckwheat) with 15% of total reads, followed by Poaceae (grasses) with 14%, *Asteraceae* (asters) accounted for 11%, Fabaceae (legumes) 10%, Brassiacaceae (brassicas), Plantaginaceae (plantain) 7%, Casuarinaceae (sheoaks) 6%, Solanaceae (nightshades) 5%. All other streptophytan families made up less than 5% each. There were significant differences between islands (Figure 6.5C, perMANOVA, r-squared = 0.05511, F = 1. 6622, p-value = 0.035).There were notable difference between the Nonsuch Island and the other two, particularly the rarity of Asteraceae on Nonsuch Island (Figure 6.4C) (Supplementary Data 6.1 S14).

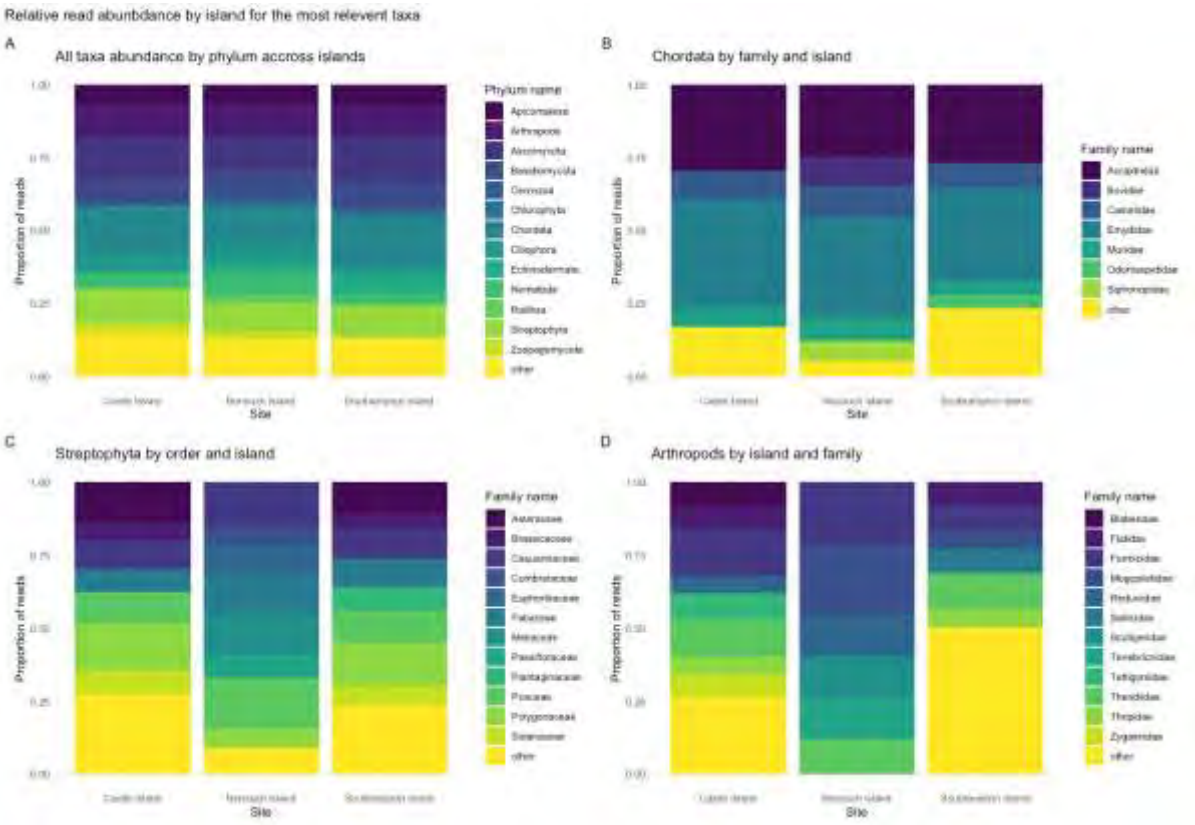

Figure 6.4: Relative read abundance of taxa by island. A) Relative abundance of the most common phyla in the dataset. B) Relative abundance of the most common diet-relevant Chordata families. C) Relative abundance of the most common Streptophyan families. D) Relative abundance of the most common diet-relevant Arthopod families.

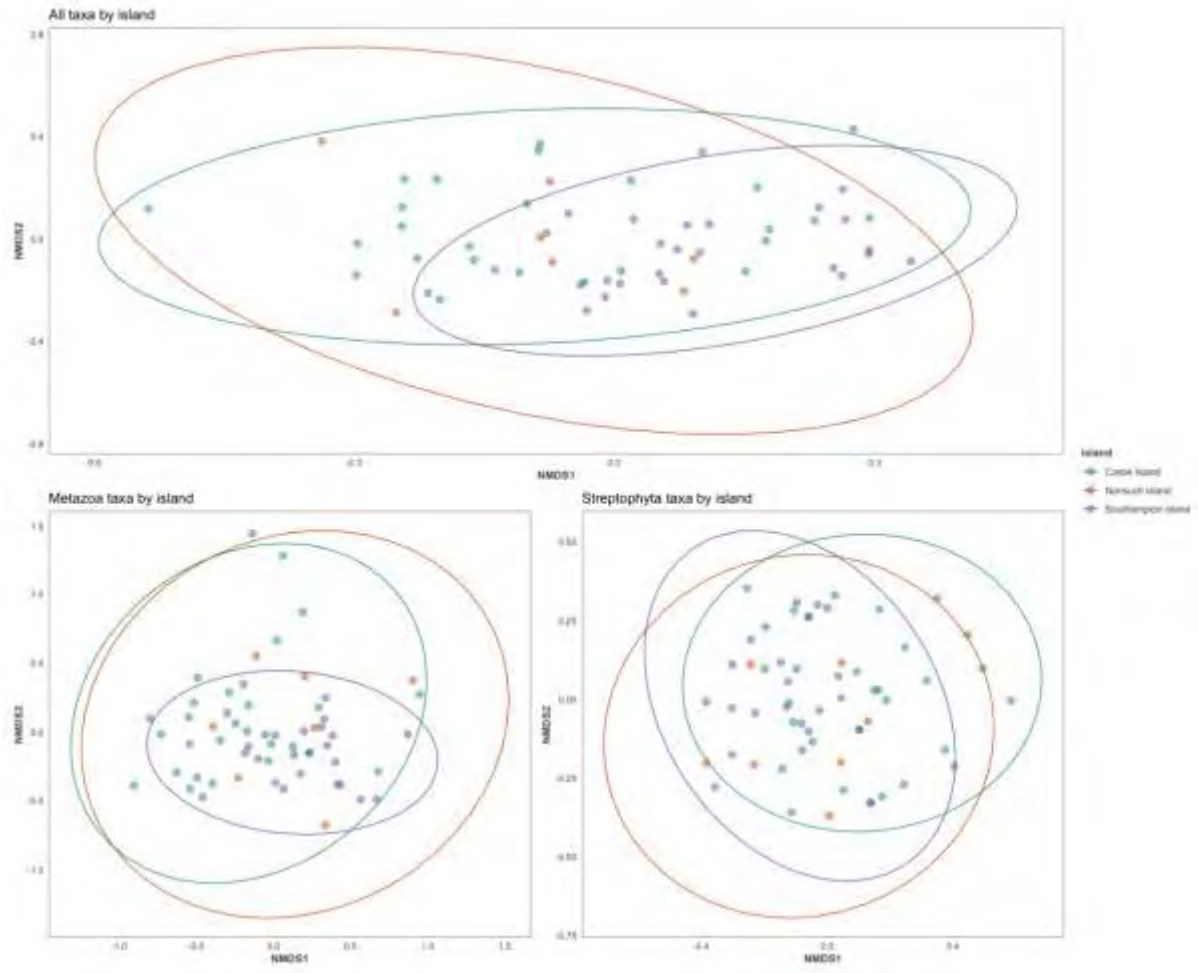

Figure 6.5: NMDS plots of relative read abundance data (Bray-Curtis dissimilarity) of skink faecal samples between islands broken down by a) All taxa (perMANOVA, r-squared = 0.068, F = 2.069, p-value <0.001), b) Metazoan taxa (perMANOVA, r-squared = 0.05, F = 1.73, p-value = 0.006), c) Streptopphytan taxa (perMANOVA, r-squared = 0.055, F = 1.662, p-value = 0.035).

Discussion

The characterisation of diet is a crucial component of any conservation effort as dietary information provides a clear outline of the ecological niche that a species occupies (Zarzoso-Lacoste *et al.*, 2016). In the case of the Bermuda skink, we knew very little about its diet prior to this study (Davenport *et al.*, 2001). We used metabarcoding to uncover the diet of the Bermuda skink, finding equal abundance of chordates, invertebrates and plants in the faecal samples, a pattern consistent across different islands.

Previous reports on the diet of the Bermuda skink are few and conflicting. Wingate (1965) reported that the main prey items of the skink were ants, woodlice and small beetles, with some scavenging behaviour. In contrast, Williams *et al.* (2022) found that the skink were reluctant to take some insect items, preferring fruit jelly, during an ex-situ feeding experiment. However, our finding that the skink has a broad omnivorous diet in the wild, with equal contributions from vertebrates, invertebrates and plants is not unexpected, given many small reptiles are omnivorous (Tercel *et al.*, 2022)The proportion of vertebrates is higher than might be expected from a small reptile but is supported by previous work. The high proportion of bird in faecal samples likely supports previous hypotheses that the skink may scavenge on the eggs of sea birds or on dead chicks (Wingate, 1965). The presence of fish in the samples, also indicates that scavenging, potentially from seabirds, is a major part of the diet.

It has previously been reported that skinks interact with litter left behind by people, which often leads to the death of skinks (Davenport *et al.*, 2001). However, we found wheat, rice and other human foodstuffs being identified in our samples. The presence of human foodstuffs in the diet is understandable given the scavenging behaviour reported by other studies and shown here. In Nonsuch Island, a nature reserve and more difficult for humans to access, the relative contribution of taxa associated with human diet was lower. The potential importance on human provided foodstuffs in the diet should also be investigated to see if its consumption is due to its easy availability or reflects a lack of resources pushing the skink to consume novel items. The fact they seem to prey on such a wide variety of foods, however, seems to point to a high degree of plasticity in their diet. This could well be a positive sign for the future of these populations because this will limit the chances that food availability is currently causing a decline in these

populations. However, there are significant population and ecosystem impacts for human-wildlife food provisioning (Cox & Gaston, 2018). Further work to understand how the skink access human foods, e.g. through litter, bin-raiding or deliberate feeding, is needed, to understand what conservation actions may be needed. There are likely more pressing concerns of habitat loss and the interaction with human detritus bringing its own dangers with reports of interactions of bottles and cans causing the deaths of many individuals (Davenport *et al.*, 2001; Stroud, Giery and Outerbridge, 2017).

One of the major concerns for the future of the Bermuda skink is the presence of invasive species, both those that provide competition for resources and those that may be predators of the skink itself. It has been previously reported that the diet of the Bermuda skink had a high degree of overlap with the invasive *Anolis sagrei* (Stroud, Giery and Outerbridge, 2017). This assessment of overlap was based on a morphological analysis of *Anolis sagrei* faecal samples, while information on the diet of the Bermuda skink was provided by the archives of the Natural History Museum of Bermuda (David B. Wingate, 1965). Our assessment of Bermuda skink diet differs substantially from that of *A. sagrei*, the latter appearing to a consumer of terrestrial invertebrates, with limited evidence that it consumes plants or scavenges. The potentially reduced overlap in diet suggests that *A. sagrei* may compete less than previously thought with the native skink. However, these differences in the importance of prey items could be due to the differences in methodology between the two studies. Traditional morphological methods may not identify remains plant material and or the scavenged vertebrates as the skink would be too small to consume bones.

There were significant differences in the diet of the Bermuda skink between islands. This was not unexpected given that the two inhabited islands sampled (Southampton and Castle) were in a different condition to Nonsuch Island, which is a nature reserve and therefore is well preserved (Turner, 2018). This differing level of human disturbance is likely to affect the species present on the islands and therefore the impact of this in the diet is logical. For example, on Nonsuch Island, seagrape, a mangrove species indicative of more natural habitats, was much more abundant than on other islands. The differences identified in the diet between the islands in this study show that it may well be necessary to create bespoke conservation strategies for each sub-population of the Bermuda skink. However, we interpret these differences between islands with caution as the number of samples for Nonsuch Island was low and we cannot therefore rely on species richness estimates for differences.

We have provided a far fuller and a more informative picture of the Bermuda skink diet than was previously known. Despite this we have also come up against some of the issues that metabarcoding studies face more generally. Firstly, 39% of our initial 98 samples were not included in the study due to no amplification. The most likely cause of this was the poor storage of the samples post-collection, despite the fact they were stored in NAP buffer (Ando *et al.*, 2020). There were also difficulties exporting the samples as they were unable to be kept in optimal conditions and this likely degraded the DNA in the samples and affected the success rate significantly. Furthermore, metabarcoding is highly susceptible to contamination meaning that the diversity present in samples can be artificially inflated, leading to inaccurate assessments about the breadth of a species diet (Zinger, Bonin, Inger G Alsos, *et al.*, 2019)(Schnell, Bohmann and Gilbert, 2015). Cross contamination can occur in several stages of sample preparation including extraction, PCR, library preparation and sequencing (Taberlet *et al.*, 2018). To account for this we included several control types including extraction, PCR and sequencing negatives to reduce the potential impact of these issues by removing MOTUs associated with cross contamination (Zinger *et al.*, 2021). Further, we curated our dataset to ensure species that were certainly not present in the study area were removed such as common carp which was present in the positive control but is certainly not present in Bermuda. When conducting a metabarcoding study marker choice has a huge effect on the results. The broader the taxa amplified by a marker the lower the taxonomic resolution to species level; therefore the ideal choice is to choose the most specific marker possible for your study (Casey *et al.*, 2021b)(Schenekar *et al.*, 2020). Because the breadth of the skink diet was unknown we intentionally selected a marker that would amplify across all Eukaryotes. This allowed us to investigate the plant diversity in the diet as well as the metazoan diversity. This comes at the expense of species resolution, and it is for this reason that 30% of our mOTUs were not identified past family level and it also leads to uncertainty about species identification more generally. The issue of low taxonomic resolution and incomplete and underrepresented taxa in reference databases affected our study in particular our marker has low discriminatory power within chordates in particular in bird and reptile groups which meant we identified these sequences with NCBI and ion most cases were unable to trust identifications beyond class level future investigations would require a revised marker design to overcome these issues.

The issues with marker resolution are compounded by any gaps present in the reference database and as a result even a perfect maker might not be able to resolve all the species

present in the samples (Schenekar *et al.*, 2020). The relative abundance can be affected by many things such as PCR efficiency marker bias and others making interpretation of such data very difficult (Deagle *et al.*, 2019). We also face the issue of distinguishing between prey items and the gut contents of those prey items. For example, we found rotifers in our dataset and it is unlikely that skinks could ever prey upon rotifers directly and these were likely gut contents of the direct predator of the rotifers (Cuff *et al.*, 2022). Therefore, metabarcoding should be used in conjunction with other studies such as behavioural observation where possible to help lend clarity to some of the ambiguities presented in metabarcoding data.

In conclusion, this study presents the most complete picture of the Bermuda skink diet yet, revealing an omnivorous and scavenging diet, with equal contributions from vertebrates, invertebrates and plants. Many of the dietary items we found are new to our knowledge such as fruit and insects that would be difficult to identify to family level using traditional methods. Metabarcoding has allowed us to see new aspects of the diet of this species the importance of fruit and the conformation of scavenging from predatory birds which was not confirmed prior to this study (David B. Wingate, 1965). This information will prove crucial to future conservation efforts both with in situ and ex situ populations. Further studies with more specific markers now that we know what the skink eats would allow us to create a more detailed picture of this species ecological niche. Finally, our information on the diet will help with the currently ongoing ex situ conservation efforts for this species.

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Chapter 7 – General Discussion

Motivation for the thesis

We are currently undergoing a period of rapid environmental change (Sih, Ferrari and Harris, 2011), which is causing species and their interactions to be in a state of flux (Zhang *et al.*, 2017). The study of predator/prey interactions has therefore become increasingly important. Diet studies have been shown to provide significant insight in describing the trophic interactions that underpin our understanding of ecosystem functionality (Deagle *et al.*, 2019), and as a result have an increasingly important role in conservation management (Thomsen and Willerslev, 2015).

Historically, diet studies have been conducted through morphological examination of gut contents either through examination of faeces or gut contents, or alternatively through direct observation (Reid *et al.*, 2013; Taberlet *et al.*, 2018). The examination of the identifiable parts of faeces requires high levels of taxonomic expertise that is often unavailable. Furthermore, even where such skill is available, the time taken to identify all the components of a faecal sample is not insignificant. There are also significant error rates and specific taxonomic groups can be difficult to account for due to the lack of identifiable remains in the faecal samples (Carss and Elston, 1996; Carss and Parkinson, 1996)These studies do however, have the advantage of requiring little specialist equipment and, with the exemption of the cost of people's time, can be conducted on a small budget. Metabarcoding of diet has been demonstrated to provide solutions to many of the problems identified with morphological studies (Shurin *et al.*, 2002; Thomsen and Willerslev, 2015; Massey *et al.*, 2021). Using universal barcodes to identify what is present in faecal samples allows the identification of many more prey identities to species and genus levels than morphological studies. Critically, metabarcoding allows the identification of species that leave little morphological evidence behind, e.g. soft-bodied invertebrates, soft tissues consumed by scavengers, or leaf matter (POMPANON *et al.*, 2012). The fact that such taxonomic resolution can be utilised without the very specialised taxonomic skills required with morphological approaches, allows the "taxonomic impediment", i.e. the scarcity of trained taxonomists, to be partially overcome. Such advantages make metabarcoding a very appealing method. Despite this, executing an effective metabarcoding experiment remains expensive, and requires

thoughtful experimental design and need for the availability of specialist equipment to be done well (Taberlet *et al.*, 2018; Liu *et al.*, 2020a).

The aim of this thesis was to devise alternative library preparation methodologies that keep the costs of metabarcoding diet studies to a minimum (Chapter 2). This would then allow them to be conducted in the absence of some of the specialist equipment that is usually required for library preparation, thus increasing the number of smaller laboratories that could undertake such projects. Added to this, the low cost of each individual library and the pooling strategy associated with the method allows the addition of multiple markers with only a fractional increase in cost. This makes hierarchical and multiple marker strategies attainable on modest budgets. Previously, metabarcoding diet studies of mesopredator or omnivore diets has been recognised as particularly difficult due to the broad nature of the diet requiring multiple markers to fully assess the composition and synthesising the information provided by multiple markers has been difficult (Tercel, Symondson and Cuff, 2021). We tested our library preparation methodology on the diet of the Bermudan skink, where almost nothing was known so we took a broad approach using a eukaryotic marker to outline the general shape of the diet (Chapter 6). We also test the library preparation methodology with multiple markers on two mesopredators, the Eurasian Otters (*Lutra lutra*), selected due to the large volume of literature on its diet (Chapter 3), and the Neotropical Otter (*Lontra longicaudis*). We specifically aimed to quantify the relative importance of vertebrates and invertebrates in their diets, something that has not previously been effectively attempted (Chapter 4 & 5).

Development and testing of a library preparation methodology

Here we developed a cost effective and robust library preparation strategy that reconstructed communities with a high level of precision (Chapter 2). We demonstrated this first using known mock communities that allowed us to test and assess for both accuracy and abundance. Although the accuracy of the community reconstruction was imperfect, the general community structure was present and, through the use of positive controls, it was possible to account for any taxonomic bias found in the produced datasets. Due to the nature of metabarcoding introducing bias at any level, it is possible

that the accuracy was affected more by the marker bias in the PCR stage (Alberdi *et al.*, 2018; Francesco Ficetola *et al.*, 2020), something supported by the consistency of the patterns between experiments on the same mock community. Methods similar to ours that have published recently have focused on the removal of tag-jump from libraries (Carøe and Bohmann, 2020). Our method does have noticeable levels of tag jump (between 2-7%). However, this is manageable and can be dealt with bioinformatically due to our robust experimental design (Zinger, Bonin, Inger G. Alsos, *et al.*, 2019). One library we produced did have significant levels of tag jump at 20% but this was very likely due to primer degradation, and we were still able to clean our data, retaining all samples. This did highlight a need to improve our primer management in the laboratory. Although the reduction in tag jump is desirable, it is more important that communities can be precisely and accurately assessed, something we demonstrate our method is capable of doing. The ability to produce such libraries in conditions outside of the ideal laboratory for metabarcoding will be valuable resource for many laboratories. Furthermore, the quality control methods presented here are applicable to other tag based ligation strategies, allowing researchers to tailor their approach depending on their specific circumstances.

The efficacy of the method developed further demonstrated through its successful implementation on the diet of three different mesopredators Eurasian Otter *Lutra lutra, Neotropical Otter Lontra longicaudis* and Bermuda Skink *Plestiodon longirostris.* In all cases, the method outlined here produced consistent results with yields and quality scores that either reached the expected outputs provided by Illumina or significantly exceeded them. The high quality of the data produced ensured that we had enough sequencing depth to draw valid ecological conclusions in each study, even after stringent data cleaning meant that many reads were discarded. The consistent results presented here, even when we selectively excluded some of the quality control steps that require specialist equipment that may not be present in some smaller laboratories, demonstrates it will be possible to produce high quality metabarcoding studies even in laboratories that lack some of this equipment, hopefully opening up metabarcoding to a wider community and helping conservation efforts more generally.

A systematic review of *Lutra lutra*.

Our systematic review of the state our knowledge of the Eurasian Otter *Lutra lutra*, highlighted significant imbalances in the literature that were both geographic and topical in nature. Diet and population size dominated the literature, making up more than half the total number of studies published, and there was comparatively little literature on *L. lutra* behaviour, contamination and health. Most of the literature was focused on a small part of the species range, namely the United Kingdom, and Western Europe more generally, with the two countries with the largest proportion of the species range, China and Russia, accounting for a very small part of the overall body of literature. The geographical imbalance highlighted is an issue for *L. lutra* conservation, as the threats facing the species in Western Europe are very different to that of Asia. While our study of *L. lutra* diet in the UK contributes to overabundance of studies of this kind in this location, the development of our method should allow the study of diet in otter populations that are more remote and less well studied.

We found that the large amount of information present on the diet of *Lutra lutra* within the UK made it an ideal location to test new methodologies for metabarcoding. Most studies that have been conducted in the UK have been done using traditional morphological methods, although more recently some metabarcoding studies have been published (Kumari *et al.*, 2019; Harper *et al.*, 2020). Both the morphological and metabarcoding studies broadly concur on otter diet, with fish being found to contribute around 80% and amphibians, birds, and reptiles making up the remaining 20%. Interestingly, despite morphological studies finding invertebrates present in the diet, none of the metabarcoding studies published in the UK have looked at invertebrate consumption, therefore potentially ignoring an important dietary component for *Lutra lutra* as we reveal (8% of diet).

Testing of a hierarchical marker strategy

The broad nature of mesocarnivore diet represents a challenge for metabarcoding as universal barcodes tend to either sacrifice taxonomic breadth for taxonomic resolution or vice versa (Srivathsan *et al.*, 2015). Therefore, if a marker encompasses the full diet of a species that consumes vertebrates and invertebrates, it is unlikely to maintain good taxonomic identification of the dietary items down to a genus or species level (Taberlet *et*

al., 2018). Conversely, a marker that can identify prey items down to such levels is unlikely to cover the full breadth of the diet. As a result of this, multiple markers are often used to investigate different aspects of a species diet, such as invertebrate, vertebrate, fish or plant markers, amongst others (da Silva *et al.*, 2019; Topstad *et al.*, 2021). However, due to the lack of overlap between such markers it is often impossible to compare the relative importance of the groups covered by each marker. This can lead to difficulties in assessing the relative importance of different dietary components, which is essential in determining the ecological niche that a species occupies, and for the development of effective conservation strategies for a species.

The importance of marker strategy was underlined by the study of Bermuda Skink (Chapter 6). Previous knowledge on its diet was scant; it has been proposed it scavenged, was predominantly an insectivore, and had a preference for fruit jelly (David B. Wingate, 1965; Garber, 1988; Stroud, Giery and Outerbridge, 2017). As we knew little about the diet, we chose a single marker to get an overview of the diet, encompassing all possible prey items; plants, invertebrates and vertebrates. We found that vertebrates, plants and insects were equally important in the diet. Despite this success, the lack of more specific taxonomic markers meant that many of the prey items could not be identified consistently, even to family level, while there were a significant number of misidentifications, especially amongst the vertebrate community. There were also a significant number of reads assigned to taxa, such as algae and fungi that were likely incidentally consumed or collected, limiting the sequencing depth available to identify prey items. Despite these issues, a more specific marker would have missed important aspects of the diet. The inclusion of plants into the diet was unknown at the beginning of the study and would almost certainly been overlooked if a more specific marker was chosen. A hierarchical strategy would have been ideal, however the cost of a multiple marker study was outside the scope of the project, making the choice of the eukaryotic marker the best compromise in the circumstances. Despite this, the limitations demonstrated by using the single broad marker for the Bermuda skink shows the value of ensuring the full breadth of a diet is studied.

My study on the diet of Eurasian Otter *Lutra lutra* attempted to resolve the compromise between marker breadth and resolution by using a hierarchical marker structure, with a Eukaryotic marker that covered the full range of *L. lutra's* diet. This marker is unable to resolve the prey items down to a species genus or species level but could be used to look

at the relative importance of higher-level groups, such as vertebrates and invertebrates. Alongside this broad marker, we chose two more specific primers, a vertebrate and invertebrate marker, that had better species level identifications, allowing for a more detailed analyses of the diet to be made. We found that invertebrates were indeed an important component of the diet in our population of *L. lutra,* constituting 8% of the diet in relative read abundance measures. This inference was made from the Eukaryotic marker. This information was then combined with the genus and species identification provided by the specific markers. We showed that the most important invertebrate prey item was the common green crab. This was an identification that was not attainable with the Eukaryotic marker as this could not identify the crab reads to this taxonomic level. In the absence of the Eukaryotic marker to provide context, it would be impossible to determine if crab or a single species of fish were more important in the diet of *L. lutra*, as the lack of shared taxa between the markers makes direct comparison impossible. Although this is not an insurmountable problem with *L. lutra* because we know that vertebrates constitute most of the diet, the lack of a higher-level marker could make it difficult to draw conclusions in other less well studied species.

With the successful testing of a hierarchical marker strategy on a well studied species in *L. lutra*, we further demonstrated the efficacy of this marker structure on a related but less well studied species the Neotropical Otter, *Lontra longicaudis*. This study revealed previously unknown aspects of the diet. We demonstrated that invertebrates were a significant contributor to the diet at 15% of the overall read abundance, as calculated with a combination of the Eukaryotic and Invertebrate markers. Again, such inferences would have been impossible without this marker structure.

These studies show that it is important to consider the marker structure when designing metabarcoding experiments. This ensures that there is a marker that covers the full breadth of a species diet, as it is essential if the relative contributions of each taxonomic group are to be effectively assessed. This is more difficult with mesocarnivores that often have a broad diet encompassing a wide variety of taxa, but we believe that hierarchical strategies, such as those presented here, may be part of the solution to such difficulties.

Monitoring of the diet and wider environment using metabarcoding.

The study of predator diets can be used to monitor and identify changes in the wider environment. This has been done many times, for example, with investigations into defaunation and the identification of invasive crayfish in faecal samples (Adrian and Delibes, 1987; Narváez *et al.*, 2020). *Lontra longicaudis* is known to predate on an invasive armoured catfish (Juarez-Sanchez, Blake and Hellgren, 2019). However, we detected the invasive catfish in the otter faecal samples at all of our sites; three of which were not known to have harboured the invasive species. This demonstration of detecting a range expansion of an invasive species highlights the power of metabarcoding diet to assess environmental changes.

The detection of invasive species was an important piece of information (Moon, Blackman and Brewer, 2015), but more generally we also revealed the breadth of the diet of *Lontra longicaudis* in Central America, something that was previously unknown. We detected three times as many species as were found in any previous diet study of *Lontra longicaudis,* and twice the number of vertebrates. The lack of guides for identifying prey items morphologically in Central America makes the prospects of finding out the depth of information we revealed about this species diet very difficult. The information relating to the diet of *Lontra longicaudis* is essential for its conservation (de Almeida and Ramos Pereira, 2017b). Currently the populations of this species are undergoing precipitous declines in Central America due to habitat loss, change of land use, contamination, and conflict with humans. Information on the diet will help with the development of conservation strategies, while the consumption of invasive species that are causing declines in commercially important native fish stocks may well help change the perception of *Lontra longicaudis* in local communities(Rheingantz, Santiago-Plata and Trinca, 2017). We also found that the otter was not eating any commercially important fish, and thus were not in direct competition with fishers..

Limitations of methods

Despite the advantages of utilizing the methods shown above, there remain some technical issues with the ligation-based methodology. Firstly, the issue of tag jump, which is an increasing cause of concern for metabarcoding studies, is also present in our

method (Bohmann *et al.*, 2022). The rate varied between 2 and 20% of the total number of reads being misassigned due to tag jump. Although this number is high, overall the number of reads of any given species assigned as a tag jump event is low. There were fewer than a few hundred reads in any given technical replicate assigned as tag jump, making filtering out rare species even more crucial when assessing diversity (Coissac, Riaz and Puillandre, 2012; Tedersoo *et al.*, 2022). The fact that this issue affects rare species that typically species that make up less than 1% of the total read count in a sample, does limit its negative effects for diet studies as faecal samples generally contain a small number of highly abundant species, and the remaining low abundance species can easily be filtered out below a given threshold. Interestingly, in the data we produced the main driver of tag jump percentage did not seem to be the blunt-ending stage of the library preparation but was more correlated with the number of uses a primer set had gone through. This makes sense, as the barcodes are present on the primers and any degradation to the primers is likely to degrade the identifying barcode. This would guarantee misassignment as the incomplete barcode is likely to be repaired incorrectly in the initial PCR stage. If this is so, the tag jump could theoretically be reduced by more stringent lab practices, reducing the number of freeze thaw cycles and the general usage primers go through in metabarcoding projects.

The marker strategies employed in this study, although powerful and allow for the full breadth and relative importance of each taxonomic group to be assessed, did come with some compromises. The use of only one primer pair for each taxonomic group (e.g. vertebrates). Each primer pair comes with its own bias, over amplifying some groups and under amplifying others (Casey *et al.*, 2021c). The use of multiple primer pairs for each taxonomic group has therefore been recommended where possible (Topstad *et al.*, 2021). In our case we did not use multiple markers as there was insufficient sequencing depth available on the sequencers we had access to, and therefore a compromise had to be reached between studying only a portion of the diet in more detail, or all the diet in less detail. Ideally this compromise would not have to be made however, financial and technical considerations are always an issue and individual studies need to weigh up what they believe is most important for answering the questions they have. In our case this was to look at the full breadth of the diet to test a broadly applicable strategy to many species.

The barcodes used in this study also suffer from the difficulties all universal barcodes face in metabarcoding. Incomplete reference databases mean many relevant reads remain unassigned or misassigned as the barcodes were not designed for the full taxonomy present, and therefore, many species may not be identifiable down to species or even family levels (Hestetun *et al.*, 2020). Ideally each metabarcoding study would have a bespoke reference database, however, this is unrealistic in many cases (Taberlet *et al.*, 2018). In biodiversity hotspots, the collection of all relevant prey items in advance would be difficult or practically impossible. The collection of such specimens would also need separate permits that would be extremely broad in nature and therefore extremely difficult to obtain. The financial cost of producing such databases would also prevent many groups from completing metabarcoding projects if this was a requirement. We therefore used, as is common, well known pre-designed markers for our study but suffered with erroneous and incomplete identifications. This limitation is a significant issue for our, and many other, metabarcoding studies.

The use of predator diet to investigate and monitor the diversity of the ecosystems they inhabit has long been a tool in ecology (Ruppert, Kline and Rahman, 2019; Harper *et al.*, 2020). The advent of metabarcoding has opened the opportunity to conduct such studies on a larger scale, thereby increasing their power to make inferences about the state of ecosystems. However, such studies always face the issue that predators are selective consumers, and that the consumption of prey items may have more to do with ease of capture and nutritional content amongst other variables, than of true abundance in the environment, therefore care must be taken when drawing conclusions that the frequency or abundance of prey items in the diet may differ significantly in the wider environment (Rheingantz *et al.*, 2012).

Future work

The development of a metabarcoding pipeline that circumvents several of the more specialized pieces of equipment required for metabarcoding will help spread the method to a greater number of laboratories by increasing its accessibility. The next stages of development of this method would be to reduce the effect of tag jump using the commercially available reagents from Illumia, and to investigate if the issue of tag jump

is more affected by the quality of the primers than the blunt-ending step alone. If this is so, then keeping tag jump below 10% is manageable and does not reduce read counts at an unacceptable level. An experiment that would put a primer set through an increasing number of freeze thaw cycles and created a separate library for each number of freeze thaw cycles up to a maximum of ten would give invaluable insight into this problem.

The mock communities used for testing the accuracy and precision of the method also uncovered some issues with the limit of detection, and the under and overrepresentation of certain aspects of the community. An experiment with several mock communities with different compositions, some of which are like each other and some of which that are very different, would allow us to test the sensitivity of the method to underlying community changes. Furthermore, in future studies it would be good to have at least two mock communities with a difference between them which is ecologically relevant to the question being asked. For example, if we felt a change in the relative abundance of two common species was being investigated between two sites, we could design two mock communities that reflect this change. If we are unable to identify such a change between out mock communities it would indicate we could not reliably say that there were no differences between those communities. Equally if we did detect a change in our mock communities and not in the real sites, it would give more confidence in our conclusions.

In the diet studies of all the three species investigated here, it would be good to include a greater variety of markers. In the case of the Bermuda Skink, more specific markers of invertebrate and plant taxa would be a logical next step in diet investigations. For the Eurasian and Neotropical Otters the inclusion of more fish, vertebrate and invertebrate markers would lead to more robust conclusions about the important dietary components, while consistent sample collections over longer periods of time would allow us to investigate the seasonal effects that are already well documented in these species.

In future, the expansion of the sample types used in conjunction with the method developed here would be a positive step. The library preparation method is applicable to any metabarcoding project and future studies looking at monitoring freshwater ecosystems could include eDNA water filtering as a common and highly insightful way of investigating the biodiversity present in rivers. The biodiversity found in the water

column compared with that found in the diet of otters would be an interesting point of comparison between the two types of sampling.

Conclusions

We developed a robust and effective library preparation strategy that is cost effective and can be used in smaller laboratories that may lack bioanalyzers or qPCR machines to validate their libraries, thereby widening the pool of scientists that can undertake this research. This is essential if conservation efforts are to be well informed during this period of unprecedented ecological change. Taking advantage of the cost savings our library preparation technique provided, we created and tested a hierarchical marker strategy that assessed the full breadth of the diet of two mesopredators, *Lutra lutra* and *Lontra longicaudis*, revealing the previously unassessed contribution of invertebrates to their diet and the incidental discovery of range expansion of an invasive species. Such strategies can be used on any species with a broad diet, such as omnivores, that have been practically problematic for metabarcoding up to now. Such species are far more common and are at least as ecologically important as those with narrower diets such as apex predators, so diet studies such as this are critical. We also used our new method to assess the in-situ diet of a critically endangered lizard, the Bermuda skink, in order to inform feeding in ex-situ populations. We revealed previously unknown contributions of plants to the skink's diet and the extent of scavengery. Our work highlights the potential for metabarcoding to inform species-specific conservation plans and wider monitoring of environmental change, and our methodology opens up opportunities to undertake this research, which is critical to conservation efforts.

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