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FOOD WEBS IN THE ERA OF MOLECULAR REVOLUTION – LIKE RESOLVING THE GORDIAN KNOT WITH A TRICORDER?

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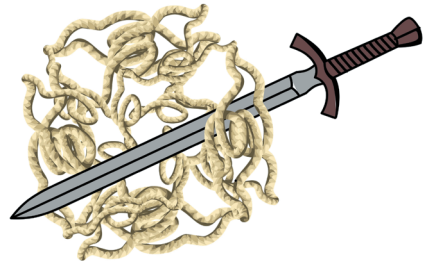
Mummulle, isoäidille, vaarille ja isoisälle.

Gordian knot

In the ancient Greece,
the Gordian knot was the ultimate challenge,
thought impossible to resolve,
until Alexander the Great (~356–323 BC)
cut the knot using his sword,
thus brutally resolving the puzzle.



Tricorder

This mystical device,
with practically unlimited capabilities,
became famous from a TV series:
Star Trek, the original series (1966–1969);
here, Mr. Data with a tricorder (ST TNG).

ABSTRACT

Living nature consists of countless organisms, which are classified into millions of species. These species interact in many ways; for example predators when foraging on their prey, insect larvae consuming plants, and pathogenic bacteria drifting into humans. In addition, abiotic nature has a great initiative impact on life through many factors (including sunlight, ambient temperature, and water.

In my thesis, I have studied interactions among different life forms in multifaceted ways. The webs of these interactions are commonly referred to as food webs, describing feeding relationships between species or energy transfer from one trophic level to another. These ecological interactions – whether they occur between species, between individuals, or between microorganisms within an individual – are among the greatest forces affecting natural communities. Relationships are tightly related to biological diversity, that is, species richness and abundances. A species is called a node in food web vocabulary, and its interactions to other species are called links. Generally, Artic food webs are considered to be loosely linked, simple structures. This conception roots into early modern food webs, where insects and other arthropods, for example, were clumped under one node. However, it has been shown that arthropods form the greatest part of diversity and biomass both in the tropics and in Arctic areas. Earlier challenges of revealing the role of insects and microorganisms in interactions webs have become possible with the help of recent advances in molecular techniques.

In the first chapter, I studied the prey diversity of a common bat, *Myotis daubentonii*, in southwestern Finland. My results proved *M. daubentonii* being a versatile predator whose diet mainly consists of aquatic insects, such as chironomid midges. In the second chapter, I expanded the view to changes in seasonal and individual-based variation in the diet of *M. daubentonii* including the relationship between available and observed prey. I found out that chironomids remain the major prey group even though their abundance decreases in proportion to other insect groups. Diet varied a lot between individuals, although the differences were not statistically significant. The third chapter took the study to a large network in Greenland. I showed that Artic food webs are very complex when arthropods are taken into account. In the fourth chapter, I examined the bacterial flora of *M. daubentonii* and surveyed the zoonotic potential of these bacteria. I found *Bartonella* bacteria, of which one was described as a new species named after the locality of discovery.

I have shown in my thesis that *Myotis daubentonii* as a predator links many insect species as well as terrestrial and aquatic environments. Moreover, I have exposed that Arctic food webs are complex structures comprising of many densely linked species. Finally, I demonstrated that the bacterial flora of bats includes several previously unknown species, some of which could possibly turn in to zoonosis. To summarize, molecular methods have untied several knots in biological research. I hope that this kind of increasing knowledge of the surrounding nature makes us further value all the life forms on earth.

TIIVISTELMÄ

Elollinen luonto koostuu lukemattomista organismeista, jotka luokitellaan miljooniin eri lajeihin. Nämä organismit ovat vuorovaikutuksessa keskenään monin tavoin; esimerkiksi pedot saalistaessaan, hyönteisten toukat syödessään ravintokasviaan, sekä toisaalta myös taudinaiheuttajabakteerit kulkeutuessaan ihmiseen. Myös niin kutsutulla elottomalla luonnolla on suuri alkuunpaneva vaikutus elolliseen luontoon eri tekijöiden kautta (muun muassa auringonvalo, lämpötila ja vesi).

Olen väitöskirjassani tutkinut monipuolisesti eliöiden välisiä vuorovaikutuksia. Näiden vuorovaikutusten verkkoa kutsutaan yleisesti ravintoverkoiksi, kuvaten lajien välisiä syömissuhteita tai energian liikettä ravintotasolta toiselle. Juuri nämä ekologiset vuorovaikutukset – tapahtuvat ne sitten lajien tai yksilöiden välillä tai mikro-organismien välillä yksilön sisällä – ovat suurimpia eliöyhteisöä muokkaavia voimia. Vuorovaikutukset ovat kiinteässä suhteessa biologiseen monimuotoisuuteen, eli lajien määrään ja lajimäärien suhteisiin. Laji kuvataan ravintoverkossa solmukohtana, jonka vuorovaikutussuhteita muihin lajeihin merkitään yhdistävillä viivoilla, linkeillä. Yleisesti on ajateltu, että arktiset ravintoverkot ovat yksinkertaisia, koostuen vain muutamista solmuista ja niitä yhdistävistä harvoista linkeistä. Tämä käsitys on juontanut juurensa ensimmäisiin ravintoverkkoihin, joissa esimerkiksi hyönteiset ja muut niveljalkaiset oli kerätty yhden otsikon alle. Kuitenkin, monissa tutkimuksissa on osoitettu, että hyönteiset muodostavat sekä lajimäärältään että biomassaltaan vallitsevan ryhmän sekä tropiikissa että arktisilla alueilla. Aiemmin haasteelliseksi osoittautunut hyönteisten ja mikro-organismien roolien selvittäminen ravintoverkoissa on molekyylimenetelmien kehittymisen myötä tuonut uusia mahdollisuuksia ekologiseen tutkimukseen.

Ensimmäisessä osatyössäni tutkin Suomessa yleisen lepakon, vesisiipan, ravintoa Varsinais-Suomen alueella. Tulokset osoittivat, että vesisiippa on erittäin monipuolinen peto, jonka pääasiallisen ravinnon muodostavat vedestä kuoriutuvat hyönteiset, erityisesti surviaissääsket. Toisessa työssäni laajensin vesisiipan ravinnonkäytön tutkimusta ajallisen ja yksilöiden välisen vaihtelun selvittämiseen sekä saatavilla olevan ravinnon suhdetta saaliiseen. Tulosten perusteella näyttää siltä, että surviaissääsket säilyttävät asemansa tärkeimpänä ravintokohteena, vaikka niiden määrä suhteessa muuhun ravintoon laskee syksyn edetessä. Ruokavalio vaihteli paljon yksilöiden välillä, tosin vaihtelu ei saavuttanut tilastollista merkitsevyyttä. Kolmannessa osatyössä tutkin laajaa ravintoverkkokokonaisuutta Grönlannissa. Löysin todisteita siitä, että arktiset ravintoverkot ovat erittäin monimutkaisia, eli lajien välillä on paljon vuorovaikutusyhteyksiä. Neljännessä työssäni tutkin vesisiipan bakteerilajistoa, sekä kartoitin löytyneiden bakteerien zoonoosi-potentiaalia. Löysin *Bartonella*-bakteereja, joista kuvasin yhden tieteelle uuden lajin löytöpaikan mukaan.

Olen väitöskirjassani osoittanut, että vesisiippa petona yhdistää monia hyönteislajeja sekä maa- ja vesiympäristön. Olen myös näyttänyt toteen, että arktiset ravintoverkot koostuvat monimutkaisesti linkittyneistä eliöistä. Lisäksi osoitin, että lepakoiden bakteerilajisto sisältää paljon ennestään tuntemattomia bakteereja, joista osa saattaa olla ihmisellekin vaaraksi. Tiivistäen, molekyylimenetelmät ovat avanneet monia solmuja biologisessa tutkimuksessa. Toivonkin, että tämänkaltainen tiedon lisääntyminen ympäröivästä maailmasta lisää arvostustamme kaikkea elämän monimuotoisuutta kohtaan.

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(I) Vesterinen EJ, Lilley T, Laine VN, Wahlberg N (2013) Next generation sequencing of fecal DNA reveals the dietary diversity of the widespread insectivorous predator Daubenton's Bat (*Myotis daubentonii*) in southwestern Finland (2013). *PLoS ONE*, 8, e82168. DOI: 10.1371/journal.pone.0082168

(II) Vesterinen EJ, Ruokolainen L, Wahlberg N, Peña C, Roslin T, Laine VN, Vasko VVW, Sääksjärvi IE, Norrdahl K & Lilley TM: What you need is what you eat? Prey selection of the bat *Myotis daubentonii*. Manuscript.

(III) Wirta H*, Vesterinen EJ*, Hambäck PA, Weingartner E, Rasmussen C, Reneerkens J, Schmidt NM, Gilg O, & Roslin T (2015) Exposing the structure of an Arctic food web. *Ecology & Evolution*. DOI: 10.1002/ece3.1647

(IV) Veikkolainen V*, Vesterinen EJ*, Lilley TM, Pulliainen AT (2014) Bats as reservoir hosts of human bacterial pathogen, Bartonella mayotimonensis. *Emerging Infectious Diseases*, 20, 960-967. DOI: 10.3201/eid2006.130956

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Contributions to the original articles.

	I	II	III	IV
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Field work	EJV TML VNL	EJV TML VVWV	CR JR NMS OG TR	EJV TML
Laboratory	EJV	EJV TML	EJV HKW EW	EJV VV
Data analysis	EJV	EJV LR	EJV HKW	EJV ATP
Writing	EJV TML VNL NW	EJV LR NW CP TR VNL VVWV IES KN TML	HKW EJV PAH EW CR JR NMS OG TR	VV EJV TML AP

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1. INTRODUCTION

Ecology is a study of interactions. These interactions are commonly described among populations or species (Naddafi & Rudstam 2013; Wirta *et al.* 2014; Saba *et al.* 2014), among individuals within a species (Araujo *et al.* 2010; Tinker *et al.* 2012; Newsome *et al.* 2015), and among microorganisms within a single individual (Schulz *et al.* 2015). The number of interacting species in the whole world is estimated to be between millions and tens of millions (Erwin 1982; May 1986, 1993; May & Beverton 1990; Ødegaard 2000; Borges *et al.* 2009; Hamilton *et al.* 2010) with the number of already known species being approximately 1.25 – 1.8 million (Stork 1993; Mora *et al.* 2011). The interactions are regulated by the features of each species, and also by the properties of the environment as a whole.

Broadly, the world may be divided into abiotic and biotic parts. The first consists of chemical and physical factors, such as sunlight, water, and ambient temperature, while the latter includes all living organisms, such as plants and animals (Hellmann 2001). Abiotic factors are crucially important as primary initiators in many species interaction networks, however, the biotic world is more commonly studied. All living organisms need to acquire energy to survive and reproduce, and indeed, the most common types of interactions are related to resource use. Furthermore, the classical theory of competition suggests that two species cannot coexist permanently using the same resource (Gause 1934), and thus, biological interactions among organisms have long been recognized as important drivers of dynamics within communities (Lurgi, Montoya & Montoya 2015). A simplified example of a network like this is a plant (*producer*) consumed by a herbivore (*primary consumer*), which, in turn, is foraged on by a predator (*secondary consumer*; Huxel & Polis 2001; Yodzis 2001). Certainly, the descriptions of natural networks are always smaller pieces of the whole network (Covich 2001). Terms like *food web* or *food cycle* are traditionally reserved for illustrative descriptions of interaction networks or the connections of energy flow.

1.1. Food webs – the Gordian knot

Based on current knowledge, the first illustration of a food web was constructed by Camerano in the late 1800's (Camerano 1880). Camerano's food web has remained poorly known to scientists, and his food web figures are very different from modern ones. The first "modern" food web was constructed some four decades later by Summerhayes & Elton (1923), after an Oxford University expedition to remote high Arctic locations, Spitsbergen and Bear Island (*Bjørnøya*). This description of the "Nitrogen Cycle" was very influential at the time of its publication, and the idea of artic food webs as simple networks has persisted through time. Before going into more detail about analysing the first and later food web studies, I shall introduce the concept of food webs in general and the most important terminology.

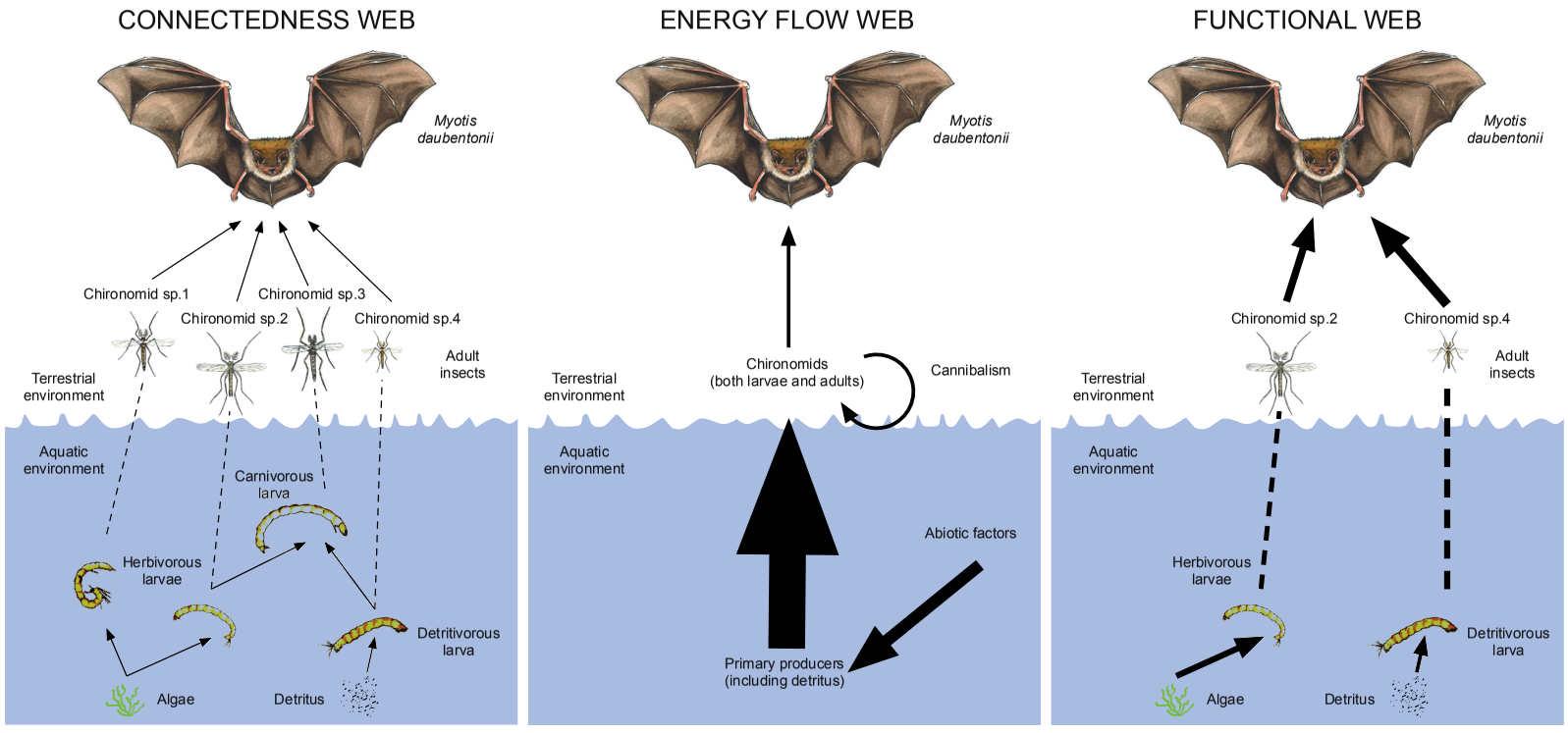

Figure 1. Food web categories. (a) The connectedness web is basically a "who-eats-who" network (pointed by arrows) with the metamorphosis of larvae into adults shown by dashed lines. In reality, *Myotis daubentonii* also consumes chironomids as pupae and a variety of other prey. (b) The energy flow web shows how the energy flows within a network; arrow thickness indicates the amount of energy passing between trophic layers. Some chironomid larvae eat other chironomid larvae, a phenomenon known as cannibalism in food web nomenclature. Abiotic factors (the temperature, sediment chemistry, etc.) affect food web energy fluctuations. (c) The functional web is based on experiments trying to pinpoint the most important species and interactions in a network. The functional web is a distilled version of the whole network, including only those species that dominate biomass or are the most influential to the energy flow in the system. All of these networks are simplified educational examples of a real food web studied in this thesis. Original drawings of *M. daubentonii* and all insects by BSc Anna Blomberg.

Food webs can generally be categorised into three types depending on the point of view of the study. First type is a classic food web showing feeding relationships (Fig. 1a; who eats whom; *the connectedness web*), the second type is an energy web (Fig. 1b; the amount of energy being transferred between levels; *the energy flow web*), and the third type is constructed through experiments to search for the most important links (Fig. 1c; which interactions are the key connections to maintain stability in the system; *functional web*; Paine 1980; Polis 1991). The first type, the classic food web, may be further divided into traditional predator-prey webs, host-parasitoid webs, and mutualistic webs (Ings *et al.* 2009). While the first two are self-explanatory, an example of the third is a flower-pollinator network, where both parts benefit of the relationship. In addition, biological interactions are often discussed in terms of generalism versus specialism.

A species (usually named in food web terminology as a '**node'**) is considered a **specialist**, if it has only few connections (**links**) to other species. Then again, if a species has many connections, it is considered a **generalist**. The ratio of realised links to all possible links in a network is also known as **complexity** (van Veen 2009). To clarify, if the community for example consists of 10 species, the number of possible undirected connections between all species is 45, assuming no connections to the species itself – cannibalism – is allowed. Naturally, if interactions have a direction – that is, there is difference between A eating B, and B consuming A – the number of interactions are doubled. If each predator in this described community consumes only one or two types of prey, the number of observed connections is low, and the community has low complexity and high rate of specialism (Fig. 2a). On the other hand, if generalism is common – each predator consumes two to three types of prey – the community is more complex (Fig. 2a). Complexity can be calculated by dividing the number of realised links with the number of all possible links, but the exact formula depends on the network (van Veen 2009).

It has been proposed that species rich communities would have a high rate of specialism due to a low number of connections, and species-poor communities having a high amount of generalism due to a high number of interactions (MacArthur 1972; Schemske 2009). Contrastingly, some studies have found low species diversity resulting in high specialism rate (Schleuning *et al.* 2012). Finally, some recent studies indicate that there is no direct relationship between species richness and the degree of generalism (Lewinsohn & Roslin 2008; Morris *et al.* 2014). Moreover, the connectance does not describe the distribution of the links. Having the same value of connectedness, one network may consist of almost separate **compartments** only weakly linked to other parts of the web (Fig. 2b; Krause *et al.* 2003; Ings *et al.* 2009; van Veen 2009). The terms 'compartment' and 'module' are sometimes used interchangeably, although the phrase 'modularity' might more suitably be defined as something more closely linked to a '**motif**', that is, a pattern that recurs in networks more often than in "an ensemble of randomized networks" (Fig. 2c; Milo 2002). Finally, in mutualistic networks, such as pollinator networks, a common concept is **nestedness** (Ings *et al.* 2009). A network is nested, when one species interacts with *n* number of species, another one interacts with only part of these *n* species, yet another one interacts with a subset the remaining species, and so on. When ordered by number of interactions in a matrix, the distribution is triangular and easily recognisable (Fig. 2d; Allesina 2012; Ulrich & Almeida-Neto 2012).

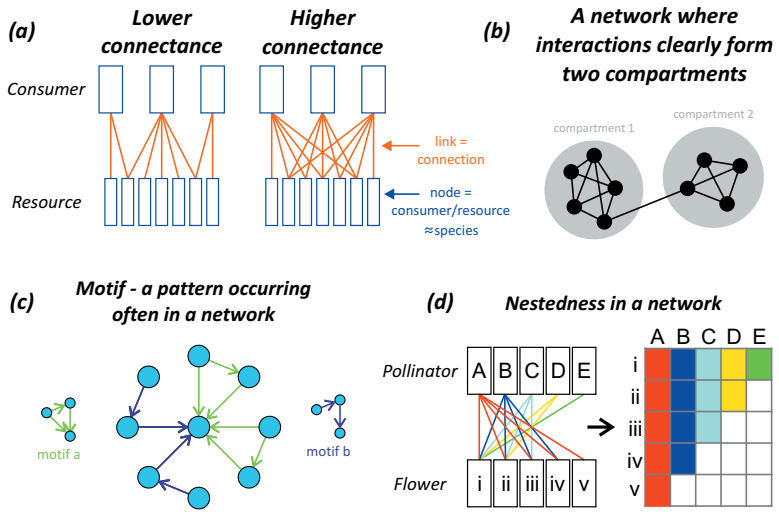

Figure 2. Different concepts in food webs. (a) Connectance networks: who eats whom. The two networks have different degree of connectance, also referred to as linkage density. Both networks have same number of consumers and resources, but the right one is more densely linked. In food web terminology, a species or any resource is a *node*, whereas any connection between nodes is a *link*. (b) A network that consists of two densely linked compartments with only one connection between the two compartments. (c) Motifs (or modules) inside a network. Motif 'a' resembles an omnivory module and motif 'b' is more like a classic food chain. (d) Nestedness: when an illustration of a mutualistic food web of pollinators and flowers (d; left) is transformed into a matrix (d; right), it is easy to see that pollinator A visits all five flowers, pollinator B visits only four of these, pollinator C visits three of these, etc. This kind of network is perfectly nested.

These concepts – specialism, generalism, complexity, compartments, motifs, modularity and so on – have repeatedly been associated with dynamics of community ecology. When specialism is high, the **modularity** may also be high (Fig. 2b). On the other hand, when the network consists of generalists, the interactions easily tap on to each other leading to low modularity (Fig. 2b). This leads us to another important aspect of food webs: **compartmentalization**. Compartmentalization means that there are distinct, more or less separate clusters inside a larger network. Compartments have been linked to network stability, but results have been inconclusive on whether compartmentalization increases stability by retaining disturbance in one compartment (Krause *et al.* 2003) or whether it decreases stability (Pimm 1979). More recently, it has been shown that compartmentalization really seems to increase stability in a food web (Stouffer & Bascompte 2011). Interestingly, species inside a compartment seem to be a) more related to other species in the same compartment, and b) more similarly sized, than all the species in the network on average (Rezende *et al.* 2009).

Generalist networks might also have other properties impacting the dynamics of populations: competition between species is not always direct, but rather indirect. Although all-embracing definitions for *direct* and *indirect effects* is difficult to construct and definitions of other variables, such as *effect* itself and *interaction* are necessary (Abrams *et al.* 1996), I will shortly explain the terms as used here. **Direct effects** are those that involve only two species and where the first species has a direct effect on

the other species. An **Indirect effect** needs at least a three species in a system, where one species (a shared predator, for example) affects one (prey) species which further, via some mechanism, affects yet another (prey) species. The study of the relationship between orca whales (*Orcinus orca*), sea otters (*Enhydra lutris*), sea urchins and kelp forests in western Alaska offers an example of direct and indirect effects. In the presence of orca whales, the sea otter abundance declined due to increased predation pressure, and as a result, sea urchin biomass increased and the kelp density declined due to increased kelp grazing by sea urchins (Estes *et al.* 1998). To simplify, the orca whale had a direct effect on sea otter abundance, but an indirect effect on sea urchin and kelp densities. When competition between species happens indirectly through a shared enemy, it is called **apparent competition** (Holt 1977; van Veen, Morris & Godfray 2006). The different concepts may be linked together through imaginary food webs: in a loosely linked web, where the specialism is high, both direct and indirect effects are mainly **vertical** (the web on the left in Fig. 2a); but in a densely linked system consisting of generalists the web structure allows also direct and indirect **horizontal** effects (the web on the right in Fig. 2a; Chaneton & Bonsall 2000; Morris, Lewis & Godfray 2004). Briefly explained, horizontal effects happen within trophic levels and vertical effects between trophic levels.

Turning the focus back to one of the first modern nutrient cycles described (Summerhayes & Elton 1923), there has been a lot of discussion of its focal taxa. While the significance of this first illustration of biological interaction web to ecological research should not be undervalued, it seemed to allocate excess weight on vertebrate organisms. Summerhayes and Elton (1923) listed approximately 20 bird species, a few mammals, roughly 50 invertebrates (including Diptera and Collembola as most species rich), fishes and a number of plants. The exact number of species is difficult to determine, since the delineation for some of them has changed during the years. Later on, Elton (1927) developed his ideas further describing a diverse set of food webs including marine networks around herring and plant-herbivore-parasitoid -system on pine trees. All in all, with the help of increasing resolution of arthropods, many more links between species (direct and indirect) have been shown in the very same locations of the first food web (Hodkinson & Coulson 2004).

From the current point of view, an increase in connections along with the increase in resolved arthropod taxa is intuitive and logical, since arthropods form the main part of animal biomass in many regions (Strong, Lawton & Southwood 1984; Wilson 1992). The well-accepted opinion in modern science is that the diversity of most taxa is highest near tropics (Gaston 2000), but on the other hand it has been proved that in the Arctic region, where species richness generally is low, invertebrates form the majority of diversity (Danks 1992; Várkonyi & Roslin 2013). To summarize the current state of knowledge of interaction networks, a food web always consists of those who produce energy to the system (autotrophs), and of those who exploit that energy (heterotrophs) although the division is not always unambiguous (Polis & Strong 1996). Secondly, food chains are usually short, that is, even top predators are only a few links away from the producers (van Veen 2009). Thirdly, in all the systems, with the same amount of species and links, there may be a variable number of separate modules inside the interaction networks,

and a different degree of nestedness. Apart from these basic rules, almost everything else we know about food web stability and complexity has now changed due to new groundbreaking methods.

1.2. Molecular revolution – the tricorder

1.2.1. The beginning of DNA barcoding

Ever since the early days of mankind, there has been an immense need to identify, classify, and categorise objects in the surrounding environment. As time passed by, this internal thirst for knowledge matured and natural sciences have evolved into several fields, such as physics, chemistry, and biology. Moreover, while the inspiration and motivation to explain the world around us has slightly changed during thousands of years, the need to describe the natural phenomena is still the same. While as sophisticated device as a *tricorder* from the fictionary Star Trek realm is still waiting to be assembled (Waters 2011), huge leaps have been taken towards such methods (Handley 2015).

In the early 2000's Paul Hebert and colleagues proposed using a short fragment of DNA to identify species (Hebert *et al.* 2003a; Hebert, Ratnasingham & de Waard 2003b). Their idea was to use a standard gene region – 658 base pairs of mitochondrial *cytochrome oxidase subunit I* (abbreviated COI; sometimes also COX-1, CO1, etc.) – that varies sufficiently between species but not much within species (Hebert *et al.* 2003b). While the proposed gene region is not suitable for all the life on earth, it has proven to be useful in many situations, especially in the study of cryptic and morphologically challenging animals and plants (for example Hebert *et al.* 2004; Schindel & Miller 2005; Kress *et al.* 2005; Greenstone *et al.* 2005). The use of short fragments of DNA to identify species became known as 'DNA barcoding' regardless of the specific gene region or taxon (Savolainen *et al.* 2005). The application of DNA barcoding to dietary research really began when a vast publicly available reference library was published (Ratnasingham & Hebert 2007). At the present, DNA barcoding is being applied to many research areas directly connected to everyday life, such as revealing unethically produced and marketed seafood (Armani *et al.* 2015), to control illegal hunting (Chen *et al.* 2015), and studying the patterns of medically significant pathogens (Irinyi *et al.* 2015).

The more unorthodox DNA barcoding studies are those that use animal remains – scat, stool, faeces, droppings, you name it – to identify species too rare to observe otherwise, to indirectly observe crop pests or to reveal dietary array of the sample "donor". Big wild cats – such as tigers, jaguars, and leopards – are well-known subjects of non-invasive faecal DNA. Even though practically everybody on the planet identifies a tiger upon encounter, for these animals, it often is difficult to obtain biological data due to small population, large territories and their conservational status. On the other hand, it is unnecessary to capture them since faecal samples offer a solution to species and sex determination (Wan *et al.* 2003; Sugimoto *et al.* 2006; Haag *et al.* 2009), identification of bacterial flora (Tu, Zhu & Lu 2005), estimation of population structure (Bhagavatula

& Singh 2006), and even identification of the diet (Farrell, Roman & Sunquist 2000). These examples highlight the advantage of faecal analysis coupled with DNA barcoding, and the method is more than suitable for other organisms that are difficult to study. Bats are such a group of animals, whose nocturnal and secretive life style brings about challenges for scientists working with them. Indeed, one special and extremely species-rich group of predators whose dietary interactions have been difficult to study before the molecular era, are insectivorous bats. On a larger scale, insects and other arthropods provide nutrition to a huge variety of animals, including many insect predators themselves (Greenstone *et al.* 2014).

Clare *et al.* (2009) were among the first to make use of the reference library to analyse the diet of the bat *Lasiurus borealis* by picking up the DNA fragments from the faecal pellets and sequencing these fragments individually. They were able to identify 127 prey species consumed by 56 bat individuals, prey items per bat varying from 1 to 7 (Clare *et al.* 2009). The method proved accurate, even producing rather long (over 600 bp) prey insect sequences, but overall it seemed a bit too laborious, since it needed dissection of each pellet and analysing the prey fragments one by one. Thus, yet another development was needed to allow more comprehensive dietary analysis. This advancement came in the form of high-throughput sequencing (HTS; also next generation sequencing: NGS) technologies, when they became vastly available during the early 2010's (Metzker 2010). These technologies differ from the traditional sequencing in that millions of reads are simultaneously sequenced from mixed samples (Clare 2014). Dietary studies for insectivorous predators took a leap forward, when a novel, short-fragment 'mini-barcode' (157 base pairs) was published and it's potential for faecal analysis was realised (Zeale *et al.* 2011). Nowadays, these primers are routinely paired with NGS in a manifold of dietary studies in bats (for example Clare *et al.* 2011, 2014a; Bohmann *et al.* 2011; Krüger *et al.* 2014a; Bobrowiec, Lemes & Gribel 2015).

1.2.2. Insectivorous animals and their prey

Bats are the second largest mammalian order in the world (next to Rodentia), and there are approximately 1200 species worldwide (Schipper *et al.* 2008). New bat species are still being described regularly all over the world, mostly in the tropics where species richness is the highest (von Helversen *et al.* 2001; Nogueira *et al.* 2012; Mahmood-ul-Hassan & Salim 2014; Csorba *et al.* 2015; Goodman *et al.* 2015)behavioural and genetic characters of whiskered bats revealed a new European bat species within the family Vespertilionidae. We describe the morphology, karyology, genetic similarity, ecology and distribution of Myotis alcathoe n. sp. It closely resembles Myotis mystacinus, Myotis brandtii and Myotis ikonnikovi in morphology, but all four species show clear genetic differences in two mitochondrial genes (ND1 and 12S rRNA. The majority of bats primarily consume insects (Hill & Smith 1984). This behaviour is reported from lower latitudes to the northernmost edges of the distribution, including all continents except Antarctica (Dietz, Nill & Helversen 2009).

Dietary studies, spanning from early 1900 to date, have so far shown that the diet of bats consists of a diverse set of prey, ranging from flying midges to ground-dwelling beetles and spiders (for more recent studies, including morphological and molecular ones, see for example Shiel, McAney & Fairley 1991; Sullivan *et al.* 1993; Beck 1994; Vaughan 1997; Flavin *et al.* 2001; Pereira *et al.* 2002; Leslie & Clark 2002; Clare *et al.* 2009, 2011, 2014a; Zeale *et al.* 2011; Bohmann *et al.* 2011; Razgour *et al.* 2011; Santana *et al.* 2011; Graclik & Wasielewski 2012; Nissen *et al.* 2013; Krüger *et al.* 2014a)”plainCitation”:”(for more recent studies, including morphological and molecular ones, see for example Shiel, McAney & Fairley 1991; Sullivan et al. 1993; Beck 1994; Vaughan 1997; Flavin et al. 2001; Pereira et al. 2002; Leslie & Clark 2002; Clare et al. 2009, 2011, 2014a; Zeale et al. 2011; Bohmann et al. 2011; Razgour et al. 2011; Santana et al. 2011; Graclik & Wasielewski 2012; Nissen et al. 2013; Krüger et al. 2014a. While the earliest molecular studies on bat diets mainly aimed creating prey species lists for various species (like Clare *et al.* 2009 for *Lasiurus borealis*, and 2011 for *Myotis lucifugus*), they nonetheless proved the power of molecular methods and provided valuable information giving deeper insight into predator-prey relationships.

However, only few of these studies have properly addressed the question of available prey versus the prey consumed although the testing of ecological theories for competition and resource use – such as optimal foraging theory – call for knowledge of not only what is foraged, but also what is available but not foraged. Leslie and Clark (2002) found that long-eared bat *Corynorhinus townsendii ingens* (previously known as *Plecotus townsendii*) consumed mainly lepidopterans even though they were not the most abundant prey available as revealed by Malaise trapping. At the same time, Pereira *et al. (2002)olive groves, and cereal steppes. The diet and food abundance were determined by faecal analysis and pitfall trapping, respectively. Overall, the diet (expressed as % frequency* reported that the diet of *Myotis myotis* contains mainly Carabidae beetles, crickets, and spiders; and the temporal variation in the diet reflected changes in prey abundance as revealed by pit-fall trapping in the habitat. These findings highlight the importance of paired studies using both consumed and available prey for bats (Clare 2014). Also, the conservation of species demands a proper knowledge of true dietary ecology, not just random analyses of the prey it is eating in the current habitat. Strikingly, before this thesis not a single study has applied modern molecular methods to analyse patterns of the available and consumed prey in natural conditions.

1.2.3. Number of reads and biomass

One general issue considered in my thesis is the relationship between the count of reads gained from sequencing and the biological quantity in the original sample. For example, if 1000 reads are assigned to species A and 10 reads to species B when sequenced from a single sample, does this difference reflect the true difference in biomass of these species in the original sample? Certainly, an important notion for the study of predator-prey relationships is the matter of qualitative and quantitative information. In a sense, all metrics of abundance (such as prey frequency based on presence or absence of the prey species in multiple samples) may be considered quantitative, but here I reserve the

term ‘quantitative’ to number of reads in a sample assigned to different prey species (in other words, read count metrics). In molecular studies on bat diet researchers commonly adopt a conservative approach considering only presence/absence (p/a) data (Krüger *et al.* 2014a; b) or frequencies based on p/a data (Clare *et al.* 2014a; Clare, Symondson & Fenton 2014b). To continue with the example above, both species (A with 1000 reads and B with 10 reads) would receive value of 1 in p/a data. If the same difference persisted through 100 samples, the frequency of both species would be 100%, even though species A would have 100,000 reads and species B 10,000. Whereas it might seem like a safe choice to hold on to p/a metrics when analysing data, it may provide very different results compared to quantitative data since qualitative data greatly overestimates the impact of rare species. As an important topic in the current and future studies of ecological interactions, these questions are discussed in a general way in this work.

1.3. Optimal diet theory and individual specialization

The optimal diet theory (ODT, or optimal foraging theory (OFT); Emlen 1966; Sih & Christensen 2001) suggests that when a predator is faced with declining prey abundances, it should adopt a generalist feeding behaviour (Singer & Bernays 2003) the behavioral basis of omnivory has not been thoroughly explored. Here we argue that understanding the basis of food mixing (i.e., eating different food types. More precisely, when the density of optimal prey falls and the encounter rate drops, predator should begin consuming all the prey including less optimal prey. It is a known fact that at the temperate region the prey abundance quickly decreases after the peak at the summer time (Speakman & Rowland 1999), but due to the lack of accurate paired studies linking actual prey availability to the prey observed it has been difficult to show how OFT works with bats. Besides, the OFT considers the behaviour of individual animals, that actually make the choice to hunt or not to hunt the prey, whereas most of the studies deal with species.

Many seemingly generalist species have been shown to consist of specialist individuals using a subset of all the resources available (Bolnick *et al.* 2002, 2003; Araujo *et al.* 2009; Thiemann *et al.* 2011). Ecologically heterogeneous individuals sharing the same environment will actively select a different division of prey (Araujo & Gonzaga 2007), a phenomenon largely ignored in ecological research (Bolnick *et al.* 2002). To make things more complicated, it is probable that real populations contain both specialized and generalized individuals (Bolnick *et al.* 2003). Although individual specialization appears common in many animals (Bolnick *et al.* 2003; Araujo & Gonzaga 2007; Araujo *et al.* 2009; Tinker *et al.* 2012; Newsome *et al.* 2015), the frequency of individual specialization in bats, and the extent to which it varies among populations or contexts, has attracted only little attention (Cryan, Stricker & Wunder 2012; but see Barclay 1985; and Fenton *et al.* 1998 for some evidence for specialization, although little discussed in these early articles). The lack of studies on bat individual specialism is at the least interesting, given the huge number of bat species among mammals. Thus, the question of individual specialism in *Myotis daubentonii* is one of the themes to be addressed in this study.

2. AIMS OF THE THESIS

The aim of this study is to examine ecological interactions in two separate systems. First, I am shedding light on predator-prey relationships between a temperate bat species *Myotis daubentonii* (Chiroptera: Vespertilionidae) and its prey. I investigate the diet of the bats at several locations in southern Finland. I implement non-destructive methods by analysing dietary spectrum straight from the DNA in bat droppings (**I**). The second chapter goes deeper into the dietary ecology of individual *M. daubentonii* bats while reducing the habitat-based variation. In this second study, I also analyse the relationship between consumed and available prey. Moreover, the study takes an even more sophisticated approach compared to the first study, exploiting a method that does not require the capture of the bats at all (**II**). The third article expands the food web into terrestrial system in Greenland where I study multiple guilds (= groups of species exploiting similar resources). In this article I study natural bird populations and their prey. The interaction-networks include spiders and parasitoid-host webs (**III**). In the fourth study I examine the limits of molecular methods by analysing all the genetic diversity found in bat faecal pellet. The aim is to get quantitative estimate for the ratio of bat DNA versus other DNA, but also to map bacterial flora in general. Furthermore, my aim is to go deeper into phylogenetics of one particular bacteria genus, *Bartonella* (**IV**).

The study questions in each article are:

(I) What does *Myotis daubentonii* actually eat? Does the diet differ between habitats?

(II) What is the temporal and individual variation in *M. daubentonii* diet? Does the prey spectrum change according to changes in prey availability?

(III) What is the level of connectance in the target community in the High Arctic, that is, does it consist of a series of distinct food chains or a well-connected web? Do the predator guilds form modules or do they blend together? Are there room for indirect interactions in the target network?

(IV) What kind of DNA does one single *M. daubentonii* faecal pellet hold? What kind of ecological interactions take place around bats? Are there zoonotic bacteria in bats and what is the role of ectoparasites in bacterial transfer?

3. MATERIAL AND METHODS

3.1. Study sites, species, and sampling

The sampling in south-western Finland took place on the mainland and the islands of the northern Archipelago Sea between 2008 and 2013 (Fig. 3). This area is the most intensively studied region for bats in Finland, including studies on ecotoxicology, population genetics, immunological research and migration surveillance (Jakava-Viljanen *et al.* 2010; Lilley 2012; Lilley *et al.* 2012b; c; a, 2013, 2014; Nokireki *et al.* 2013; Laine *et al.* 2013; Rydell *et al.* 2014). Studies I, II, and IV focused on the bat *Myotis daubentonii* Kuhl 1817 (Vespertilionidae), which is a small, widespread and common Eurasian bat (Fig. 4). In Finland, it reaches the northernmost edge of its distribution. *M. daubentonii* is one of the most abundant bat species within its range, and its population sizes are currently increasing (Bogdanowicz 1990; Stubbe *et al.* 2008). The species is considered a facultative seasonal migrant, covering middle range distances between summer and winter roosts, often within a distance of 100–150 km (Hutterer *et al.* 2005). However, during and between breeding seasons, *M. daubentonii* shows strong roost fidelity (Nyholm 1965; Parsons & Jones 2003). Most *M. daubentonii* bats forage over water or in the vicinity of water, but individual animals can hunt in forests, parks or meadows, usually under 1 km away from the roost (Fenton & Bogdanowicz 2002; Parsons & Jones 2003).

For studies I and IV, all the samples were collected while handling the captured bats. Bats were caught with a combination of mist nets and harp trap. Faecal pellets were collected either from laundered, individual handling bags or directly from the bat while handling and placed into tubes filled with ethanol and subsequently stored at -20 °C. For study IV, several types of samples were collected, including ectoparasites living on the bat and blood samples.

In study II, I concentrated on *M. daubentonii* colony living in an old water mill along the river Aura in order to avoid locality-based variation in diet. The faecal sampling was conducted simultaneously with insect trapping in a passive manner. To assess insect availability, we used two widely used and efficient trap types: emergence traps (similar as described in Lilley *et al.* 2012c) and Malaise traps (Malaise 1937; Townes 1972). Based on activity monitoring of bats around the mill (data not shown), we set our traps downstream from the mill. All traps were operated from August to early September in 2013, being emptied daily. The insect material was stored in >90% ethanol at –20 °C until processed. Simultaneously with insect trapping, we collected bat droppings from the colony roosting site. To avoid contamination from older droppings, we placed multiple clean paper sheets below the resting bats and then collected the fresh droppings on a daily basis while replacing the papers with clean ones. We aimed to collect at least 20 droppings per day. The collected droppings were stored in ethanol and subsequently stored in -20 °C until processed.

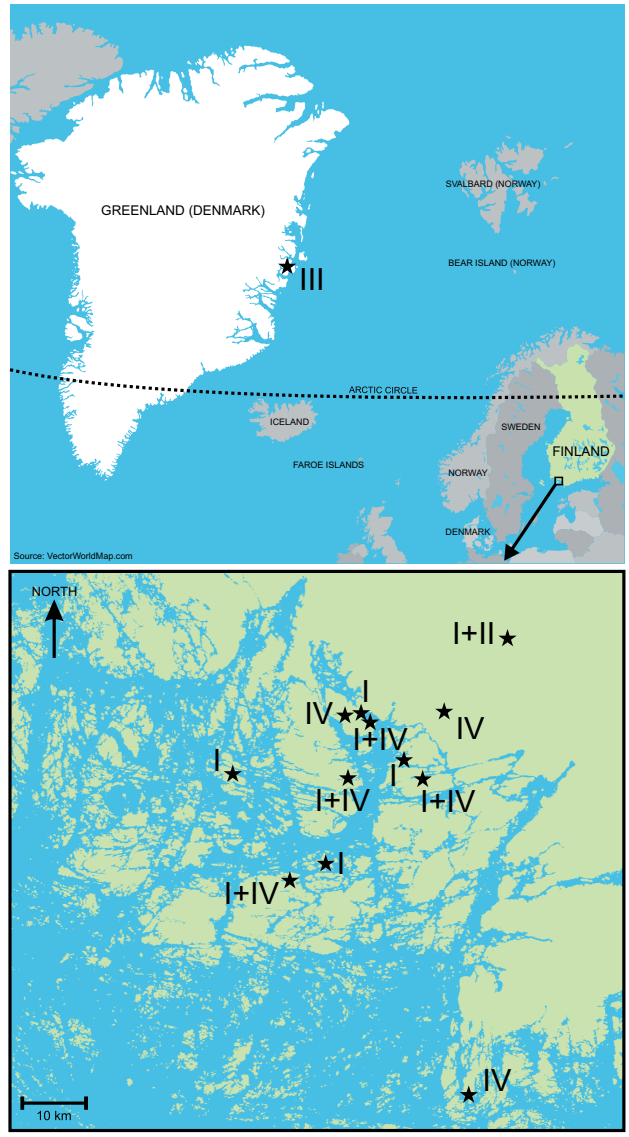

Figure 3. The sampling sites in this thesis. Study III was carried out in Zackenberg Valley, Greenland, while studies I, II, and IV took place in southwestern Finland. The Roman numbers refer to original articles.

Third study was conducted beyond the Arctic circle, approximately 1200 km west from the Bear Island, the very source of the concept of modern food web. Zackenberg Valley (74°30'N/ 21°00'W) is located within the Northeast Greenland National Park (Fig. 3). In this location, a vast effort has been invested to map the high Arctic food web in great detail (Várkonyi & Roslin 2013; Roslin *et al.* 2013; Wirta *et al.* 2014, 2015; Morris *et al.* 2014). A minority of the samples were also collected at the nearby locality of Hochstetter Forland (75° 9' N/ 19° 45' W). The documented arthropod fauna from Greenland consists of approximately 360 species. Spiders form the dominant arthropod predators in the absence of ants and ground beetles. The most species-rich order in the area is Diptera, with close to 170 species, while Hymenoptera is the second largest (59) and Lepidoptera the third (21 species; Helena K. Wirta, *pers. comm.*). Based on the number of individuals,

Diptera is indeed the most abundant order of the region (Høye & Forchhammer 2008), and Lepidoptera the locally dominant group of arthropod herbivores (Roslin *et al.* 2013).

Figure 4. The main focal species in studies I and II, *Myotis daubentonii* (Vespertilionidae), hunting over a small lake in southern Finland. A calm water surface is the favorite foraging habitat of the bat. This bat's mouth is open for echolocation calls. The photo is taken using strobe-lightning technique with 1/5 second exposure time allowing the capture of fast movements of this fast nocturnal predator. Picture by Mr. Risto Lindstedt.

To investigate and resolve multi-trophic interactions between the most influential predators and prey species, faecal samples from three abundant arthropod-feeding bird species were collected (Hansen, Hansen & Schmidt 2011): (Charadriiformes, Scolopacidae) *Calidris alpina* (L.), *C. alba* (Pallas) and (Passeriformes, Emberizidae) *Plectrophenax nivalis* (Linnaeus) (Fig. 5). In summer, these birds mainly consume arthropods (Cramp & Simmons 1993; Piersma, Van Gils & Wiersma 1996){Cramp, 1983 #2258}. Bird droppings were collected while handling the birds or from birds seen defecating. Droppings were placed ethanol and subsequently stored at –20 °C until processed. To obtain adequate number of samples for analyses, samples from *C. alpina* and *C. alba* were also collected at the nearby locality of Hochstetter Forland.

Five abundant spider species was included in the food web, representing all four families encountered in the study area; (Lycosidae) *Pardosa glacialis* (Thorell), (Thomisidae) *Xysticus deichmanni* Sorensen and *X. labradorensis* Keyserling, (Dictynidae) *Emblyna borealis* (O. Pickard-Cambridge) and (Linyphiidae) *Erigone arctica* White. The spiders were caught by live-catching pitfall traps or by visual search and manual collecting. For lepidopteran parasitoids, the material studied by Wirta *et al.* (2014) was relied on. These

specimens were caught by live-catching pitfall traps, hand-netting and visual search. The species included all abundant lepidopteran parasitoids of the region, as well as the vast majority (22 of 33) of the total lepidopteran parasitoid species (Várkonyi & Roslin 2013; Wirta *et al.* 2014; G. Várkonyi *pers.comm.* 2014).

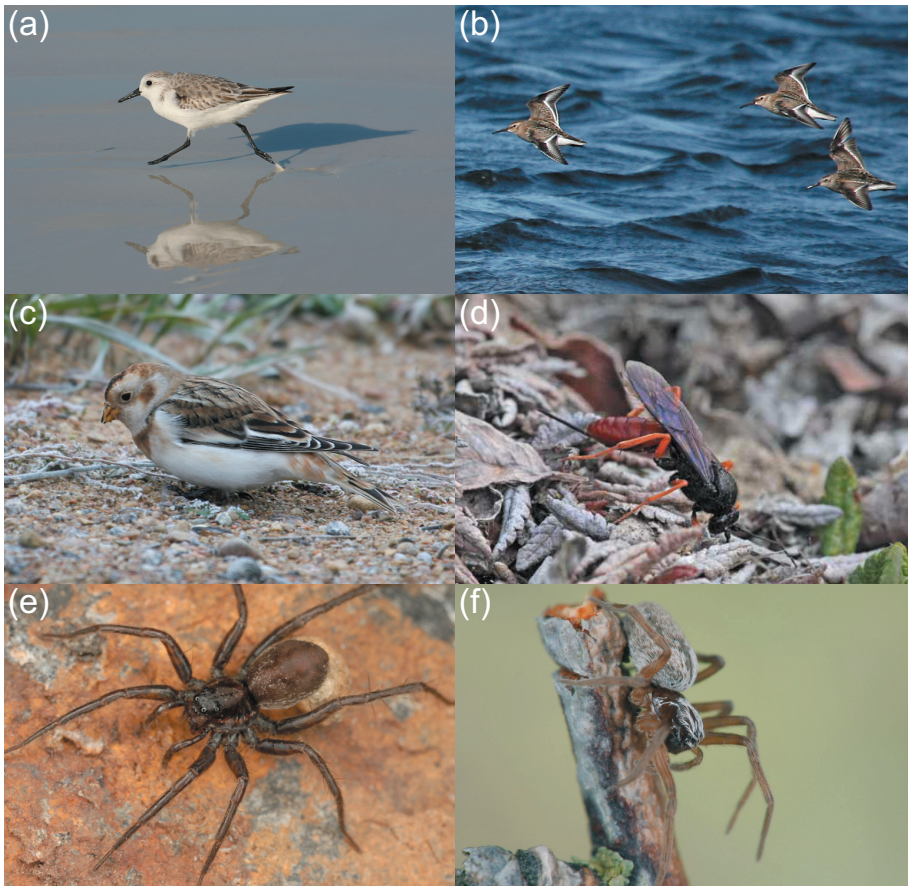

Figure 5. Some of the main study species in the third study. (a) *Calidris alba*, (b) *Calidris alpina*, (c) *Plectrophenax nivalis*, (d) a parasitic wasp *Cryptus arcticus*, (e) a wolf spider *Pardosa glacialis*, and (f) a dictynid spider *Emblyna borealis*. Bird pictures by Kari Kaunisto; wasp picture by Gergely Varkonyj; and spider pictures by Jørgen Lissner.

3.2. Laboratory work – overview of the methods applied

To accomplish the task while keeping the promises set above, I used the most recent molecular methods either recently invented and modified or further developed them if necessary. The methods include DNA barcoding, metabarcoding, metagenomics and microsatellite genotyping (Fig. 6).

As a clarification, **DNA barcoding** refers to identification of organism based on short fragment of nucleic acids, whereas **metabarcoding** means sequencing and identification of multiple targets from a source containing a mix of samples (Fig. 6). **Metagenomics** (here) denotes the sequencing of a sample as such without any prior

selection or amplification (for example locus-specific PCR) applied to the sample (Clare 2014), and finally, **microsatellite genotyping** is a traditional but still widely used method to genetically identify population structures and individuals taking advantage of short repeat-sequences present in genome (Ziegle *et al.* 1992).

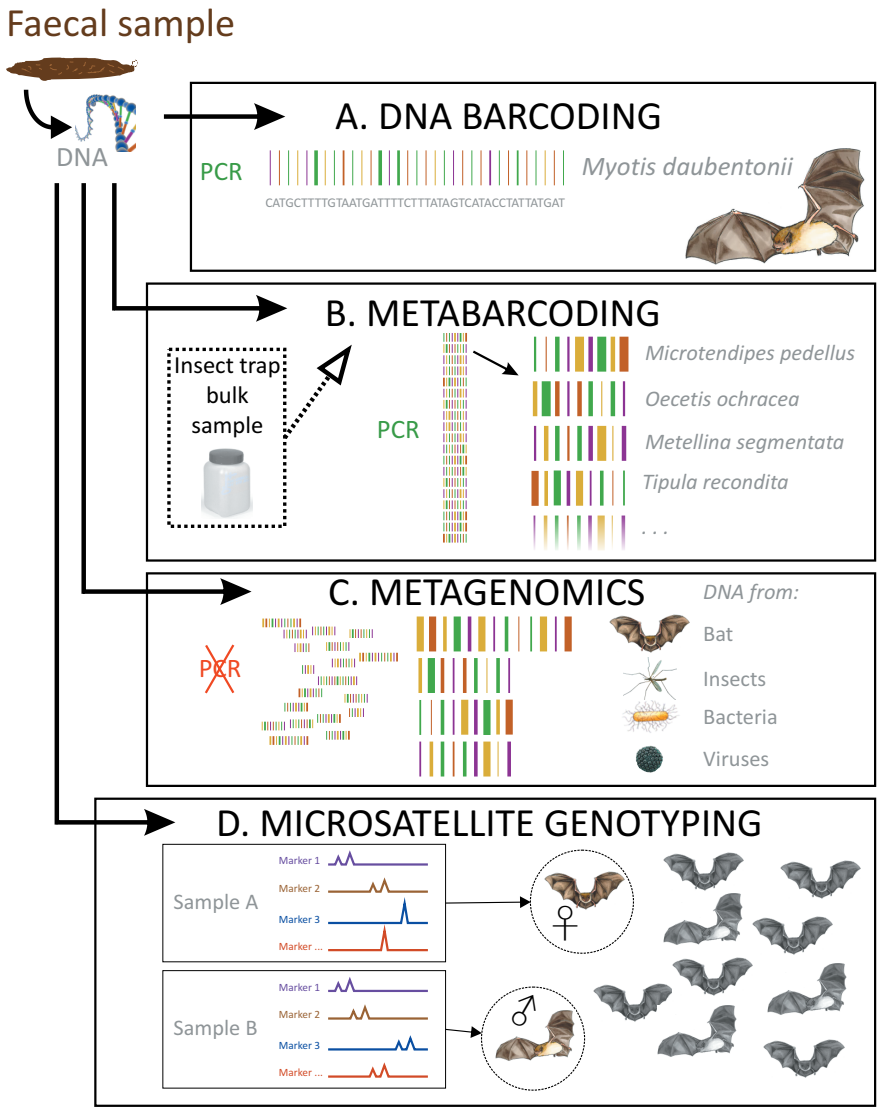

Figure 6. The molecular methods applied in my thesis. (A) DNA barcoding refers to sequencing of a PCR product, which is then compared against a reference database. (B) Metabarcoding is DNA barcoding for an environmental sample (*sensu lato*): millions of copies from pre-chosen gene regions are sequenced in parallel. (C) Metagenomics differs from metabarcoding only that the gene region is not chosen, but instead everything is sequenced as such without PCR amplification. Metagenomics from bat faeces produces DNA sequences from the bat itself, prey insects, bacteria and viruses. (D) Microsatellite genotyping is fingerprinting of individuals based on short repeat regions in DNA. If the individual has been genotyped before, the faecal pellet can be linked to that genotype. Otherwise pellets can be clustered based similar genotypes. Also, the sex and relationships within a bat population or family can be determined.

During this thesis I used molecular methods to identify arthropod species in various types of samples, including faecal droppings (**I, II, III, IV**), whole insect samples (**II, III, IV**), bulk insect trap samples (**II**), blood samples (**IV**), and cultivated bacterial strain DNA (**IV**). Even though all of the methods relied on DNA molecules, different approaches were chosen depending on the study question.

In the first study, the approach was rather simple: faecal DNA was extracted from collected samples using the Qiagen Stool Mini kit following the manufacturer protocol and a short (157 bp) COI fragment was amplified using generic arthropod markers (Zeale *et al.* 2011). Subsequently, I attached sequencing adapters including index tags to PCR products (Meyer & Kircher 2010), quantified the DNA concentration in the resultant adapter-ligated samples, which then were pooled in equimolar ratios. Negative control samples were used in all the steps showing no contamination of the reagents used. The library preparation was done in two separate batches which were sequenced using two Ion Torrent PGM 314 chips (Rothberg *et al.* 2011).

For the studies II and III, I used a wider variety of methods. Bat droppings were extracted as in the first study, but insect trap samples were extracted using a salt-extraction method (Aljanabi & Martinez 1997) modified for larger volumes. Bird faeces were extracted using Zymo Research Faecal Mini kit following manufacture's protocol. The spider samples were extracted using Qiagen Animal DNA and Tissue kit following standard protocol for animal tissue. All the different samples – bat faeces, bird faeces, insect trap samples, and spider samples – were prepared using a fast library preparation method know as modular tagging (Clarke *et al.* 2014). The principle for this method is to use special linker-tagged PCR primers in the first, 'locus-specific PCR', and then attach platform-specific sequencing adapters (having the same linker-tag) in the second, 'adapter PCR'. Adapters included special indexes enabling the pooling of several samples for the same sequencing library. The method was slightly modified from Clarke *et al. (2014)*, mainly, the locus-specific and adapter PCR were carried in separate reactions. Subsequently, the four libraries were sequenced using four Ion Torrent PGM 318 chips.

As metabarcoding requires at least approximate *a priori* knowledge of target taxa in order to design taxon-specific gene markers, metagenomic sequencing may produce data from any source material and practically any taxa can be sequenced at once. This method is commonly applied to environmental DNA, such as soil or water samples, for example in the famous study from Sargasso Sea (Tringe & Rubin 2005) and a recent study that revealed a large amount of unknown single-stranded DNA viruses from the oceans (Labonté & Suttle 2013). Metagenomics is especially good at revealing bacteria and other microorganisms, since the majority cannot be cultivated in the laboratory (Schloss & Handelsman 2005). In the study IV, I wanted to get a broader look at the DNA in the bat droppings. The method used was the same as in the first study, except the DNA was not subject to amplification; instead, the extracted faecal DNA was directly prepared for sequencing via adapter-ligation as in Meyer & Kircher (2010). The metagenomic library was then sequenced using one Ion Torrent PGM 314 chip.

In study II, each faecal pellet was genotyped using ten microsatellite loci adopting the markers and methods from Laine et al. (2013), with the following modifications: first, two microsatellite markers (E24 and D9 from Castella & Ruedi 2000) were omitted,

but instead, three X-chromosome-linked markers (Mcart and Nlgn from Frith 2010; Paur03 from Jan *et al.* 2012) were added to increase the accuracy of individual sexing. As genotyping using non-invasive samples has proved challenging (Taberlet, Waits & Luikart 1999; Buchan *et al.* 2005; Arandjelovic *et al.* 2009), I also used fresh wing biopsy punches and parallel faecal DNA samples from identified bat individuals collected from the study location as positive controls. The microsatellite haplotype replicates produced were collapsed into consensus haplotypes using software Gimlet (Valière 2002). All faecal samples were then clustered into genotypes using R package 'allelematch' with settings based on pre-analysis as described in the manual (Galpern *et al.* 2012). Moreover, I identified the sex of the bat producing each individual pellet. Samples with uncertain sex identification were left out of sex-specific analyses.

3.3. Bioinformatics – from reads to species

The developing and applying tools to computational analysing of biological data is referred to as bioinformatics. In my thesis, I used several bioinformatics tools for trimming, clustering and analysing the molecular data. All the data handling was carried out at the servers of CSC - IT Center for Science, Espoo, Finland (www.csc.fi).

The raw reads were trimmed for quality, short reads were discarded and remaining reads were subsequently collapsed into unique haplotypes independently for each data set using software Geneious (http://www.geneious.com, Kearse *et al.* 2012), FASTX tools (*FASTX Toolkit http://hannonlab.cshl.edu/fastx_toolkit/index.html by Hannon Lab*), and FASTQC (Andrews 2015) USEARCH algorithms (Edgar 2010). OTU (operational taxonomical unit) clusters were created using USEARCH (Edgar 2013) except for studies I and IV which clustering step was omitted. Assignment to species was done by using BLAST algorithm (Altschul *et al.* 1990), by sending reads directly into BOLD identification engine (Ratnasingham & Hebert 2007) or by purpose-written Python script package 'bold-retriever' version 1.0.0 (freely available at http://bold-retriever.readthedocs.org/) which uses of the BOLD ID engine but is designed for large datasets.

For ecological statistical analysis, I used several software and tools. To analyse dietary diversity in order to reveal the effect of rare and common prey species and to rarefy or extrapolate my data, I used program EstimateS (Colwell 2013). To determine differences in the species composition of faecal samples, and samples from emergence traps and Malaise traps, we performed a principal coordinates analysis (PCoA), based on Bray & Curtis dissimilarity (Bray & Curtis 1957; Legendre & Legendre 2012). To further resolve the effects of sample type, sampling day, sex, and bat individuals on variation in species composition, we conducted a permutational multivariate analysis of variance (Anderson 2001), using 999 random permutations to asses statistical significance. These analyses were done using the 'vegan' library in R (Oksanen *et al.* 2013). The species assignments were visualized using MEGAN software (Huson *et al.* 2007) and the architecture of food webs was visualized by package 'bipartite' in program R (Dormann, Gruber & Fründ 2008; Dormann *et al.* 2009). The food webs were analysed for compartments and modules using either the package 'bipartite' or program MODULAR (Marquitti *et al.* 2014).

4. RESULTS AND DISCUSSION

In the first chapter I studied the general dietary patterns of the bat *Myotis daubentonii* in various locations in southwestern Finland (**I**). While the study served as a "proof-of-concept", it shed light on many important topics regarding predator-prey interaction and proved that *M. daubentonii* is an important predator linking aquatic and terrestrial food chains. The second chapter indicated that *M. daubentonii* individuals are highly specialized predators consuming only part of the available food spectrum (II). The third chapter combined data for multiple studies to describe one of the most detailed illustrations of food webs ever built (III). The former proposals of arctic food webs as simple structures do not seem justified based on our findings (III). The fourth chapter took the whole study of interactions into a new, deeper level (IV). I made use of a method known as metagenomics to simultaneously sequence all the DNA fragments found in faecal droppings of *Myotis daubentonii*. These include bat gut bacterial flora, possible pathogenic bacteria that dwell in the bat body as reservoir, and other micro-organisms (DNA viruses and bacteria) that originate from bat's dietary insects. My results show that bat's bacterial flora and the role of ectoparasites should be taken into account when considering threat for humans via zoonosis (IV).

4.1. The first steps towards a food web (I, II)

Myotis daubentonii is a versatile predator, clearly shown by the high number of prey species found in our studies (128 and 59 prey species; study I and II, respectively). The majority of the diet consists of the order Diptera. These general findings are in accordance with previous studies (Vaughan 1997 and references therein; Krüger *et al.* 2012, 2014a; Nissen *et al.* 2013). The frequency of very few prey species was high, whereas most prey species were observed only once (I).

I found Lepidoptera to be very frequent prey (30.5%; I), which somewhat contradicts earlier studies, except one study, where frequency of lepidopteran moths (33%) was similar to my study (Taake 1991). Due to the relatively small number of studies and variation between habitats in my own study (I) it is difficult to draw definitive conclusions about the importance of Lepidoptera in the diet of *M. daubentonii*. What makes this particular case interesting, is the fact that when considered by frequency, the most important dietary taxa were Diptera (43.5%), the second Lepidoptera (30.5%), and the third Trichoptera (8.4%) but when taking into account the abundance of sequencing reads assigned to each taxon, the result changes radically: 55.9% for Diptera, 29.7% for Trichoptera, and only 7.4% for Lepidoptera (see chapter 'Linking consumed biomass and DNA read counts' for further discussion about read counts as proxies for biomass).

The first study was conducted in nine different locations, of which eight were roughly similar habitats by the seashore and one by the same river as study two. The diet of the sampled bats varied, but since the samples were temporally wide apart, the variation (both temporal and spatial) could not be tested reasonably in the first study. All in all, the most abundant prey for *Myotis daubentonii* in the majority of studies are non-biting

midges (Chironomidae). The aquatic life style of chironomid larvae makes them an important link between water and terrestrial systems (Lilley *et al.* 2012c; a, 2013). This study system is indeed ecologically, energetically, and conservationally an excellent example of a seemingly simple food chain.

To build up a wider web of interactions, we need to know who eats who or what, and to further map how energy transfers between trophic layers, we need to know trophic layers from producers to top predator (Pimm, Lawton & Cohen 1991). In my second study, I focused on one well-defined *Myotis daubentonii* group inhabiting an old water mill along a river to eliminate habitat-based variation for the diet. The food chain leading from aquatic chironomid larvae to bats is relatively short (Clare *et al.* 2011): most of the chironomids found in the diet *M. daubentonii* in Finland are either detrital collectors (approximately 50 % of observed dietary species), carnivores foraging on other chironomids or small invertebrates (30%), algae grazers (15%), or obligate miners (10%; Lindegaard 1997; I, II). Algae are important primary producers in aquatic ecosystems (Ramaraj, Tsai & Chen 2014), but also detritus is a major energy source for lower trophic layers (Shieh, Ward & Kondratieff 2002; Poepperl 2003). Although small in size, chironomids are extremely abundant in many environments, especially in freshwater habitats, reaching a larval density of 50 000 individuals per m^2 (Lindegaard 1989). This makes them very important trophic pathway to higher levels (Shieh *et al.* 2002). From the bats' point of view, the drawback in the high abundance of chironomids is the short life-span in adults; who only survive from hours to a few days, at maximum two weeks (Lindegaard 1989). Thus, the nutrition strategy of bats relies on continuously hatching chironomids.

The results from the first two studies show that *Myotis daubentonii* bats are on top of densely linked food webs, mainly fuelled by detritus consumed by chironomid larvae. The energy to the system likely comes from fallen leaf material in the river system and from the common reed decay in the Archipelago Sea shore system. These food webs seem very stable since chironomids may also thrive in a polluted waters providing sufficient nutrition for bats (Lilley *et al.* 2012c). Moreover, it has been shown that environmental toxins may transfer from aquatic system to terrestrial (Lilley *et al.* 2012a). However, specifically *Myotis daubentonii* studies show no oxidative damage to bats induced by these toxins (Lilley *et al.* 2013).

4.2. Temporal and individual variation in the diet of bats (II)

There is evidence of temporal shift in the diet of some bats, such as *Myotis lucifugus* in Canada, where dipteran insects where replaced by Ephemeroptera in the late season (Clare *et al.* 2011). Another indication of temporal shift in the diet of the same bat species (also in Canada) was published by Clare *et al. (2014a)*, who reported a minor shift from Diptera to Lepidoptera in late season for some study locations. Although these contradicting results were produced using molecular methods, there was a fundamental difference between them: the first was carried out as 'pick-a-leg' -method and the latter by direct processing of faecal pellets (Clare *et al.* 2011, 2014a). There is, however, also an ecological explanation for different dietary patterns. As Clare *et al.* (2014a) pointed

out, there is significant difference in the diet within a bat species between habitats. The same pattern of variable diet for *Myotis daubentonii* between sites was also indicated by the results in my first chapter (I).

Individual specialization is quite a common phenomenon in nature (dit Durell 2000; Bolnick *et al.* 2003; Araujo & Gonzaga 2007; Araujo *et al.* 2009; Thiemann *et al.* 2011). For bats, these patterns have been little studied and very few accurate studies about individual diet specialization in bats exists (Johnston & Fenton 2001; Cryan *et al.* 2012). In my study, there was no significant variation between individual bats and between sexes (II). Certainly, this question needs to be further assessed in later works, using more samples per individual across seasons and years following the guidelines by Araujo *et al. (2011)*.

Along with the bat prey analysis, I observed prey availability to find out whether *M. daubentonii* forages opportunistically on whatever is available, or whether there is selection towards certain prey groups. My results show that *M. daubentonii* feeds mainly on chironomids, even towards the autumn when the abundance of chironomids declined and the abundance of black-flies (Diptera: Simuliidae), an alternative prey, increased substantially (II). Throughout our sampling period, the availability of the prey, as revealed by our insect trapping using four insect traps, was different from the prey consumed.

4.3. From single-predator studies to ‘big picture’ (III)

Traditionally, information for biological interactions was gained mainly by observing and cataloguing diversity (Summerhayes & Elton 1923; Elton 1927). Gradually, scientists understood the value of experimenting, leading to specific notions on food chains and competition (Ings *et al.* 2009). A general learned opinion at the moment is that an ecological research perspective should focus on larger networks instead of describing supposedly separate food chains or interactions, and the current methods offer tools for that kind of study (Clare 2014). At the present, the focus is turning into highly resolved food webs, including several trophic layers and functional guilds (Várkonyi & Roslin 2013; Roslin *et al.* 2013; Wirta *et al.* 2014, 2015). Still, the first step in building a food web is usually to analyse the *a priori* most important predators and prey. After this, the food chain may be expanded to lower and higher trophic levels. Finally, the different subsystems may be combined to a network containing as many nodes and links as possible.

In the third chapter I expanded the study spectrum from one predator in a temperate region to a less species-rich region: Greenland in the High Arctic. The study site has been under passionate study during the last decade and the majority of terrestrial mammals, birds, and arthropods have been catalogued (Várkonyi & Roslin 2013; Roslin *et al.* 2013; Wirta *et al.* 2014, 2015). Despite the thorough investigation, the complete picture of the Arctic web complexity cannot be drawn without linking different food chains or modules together. In this study, I described the first highly resolved food web from the High Arctic combining several predator guilds (III).

There was no compartmentalization in the web, which was confirmed by statistical analysis producing non-significant results. Two parasitoid-host chains (each including one parasitoid and one Lepidoptera host) formed motif patterns. These motifs may not be real, since this part of the network remained poorly sampled with only one host larva per each chain. My results revealed many predators sharing various dipteran and lepidopteran prey species, but differently for distinctive predator guilds (III): different spider predators had similar prey spectrum, but birds consumed more lepidopteran than dipteran prey. Moreover, I found interactions between and within predator guilds; birds ate Lepidoptera parasitoids and spiders, spiders foraged other spiders, and both birds and spiders also consumed other taxa (III).

Firstly, the web constructed in the third chapter proved the Arctic network to be a truly complex system, despite the common misbelief based on low-resolution food webs. Secondly, the food web described here is one of the most thoroughly revealed terrestrial food webs in the world, thus there are no other highly resolved food webs of Arctic zone for comparison. This Arctic network results from applying a wide set of molecular methods to increase the resolution over traditional studies. Moreover, being generally well connected, the effects of indirect and direct interactions might be significantly stronger than earlier expected. Overall, the results of this study call for similar investigations around the Arctic zone and also elsewhere on the globe.

4.4. Bacteria in interaction networks (IV)

The role of microorganisms, such as bacteria, in ecological interaction networks is largely neglected (van Veen 2009). This is interesting, since there are enormous amounts of bacteria and other microbes living everywhere, including soil and sea floor (hundreds of thousand trillion trillions; Whitman, Coleman & Wiebe 1998; Schloss & Handelsman 2004; Kallmeyer *et al.* 2012).

There have been some early attempts to integrate bacteria into food webs using experimental setup (Cochran-Stafira & von Ende 1998) or in natural conditions (Hall & Meyer 1998). Later studies have compared phytoplankton-based and bacteria-based food webs in pelagic (Berglund *et al.* 2007) and riparian systems (Joaquim-Justo 2006). All of these studies found bacteria to an important trophic actor in the studied system. In the fourth study, I investigated the microorganisms found in the faecal DNA of *Myotis daubentonii*. These include the gut microbiota of bats, bacteria and viruses originating from prey insects, and a plethora of potential pathogens present in bat tissues. Following the observation of *Bartonella* spp. – a pathogenic gram-negative bacterium genus (Pulliainen & Dehio 2009, 2012; Lin *et al.* 2010) – DNA in the faecal remains, we were able to expose the presence of several *Bartonella* species in the *M. daubentonii* body. Subsequently, we confirmed that bats are reservoir of potential human pathogenic bacteria, including a pathogen *Candidatus* Bartonella mayotimonensis known to cause endocarditis in human (Lin *et al.* 2010).

Furthermore, using multilocus sequence analysis (MLSA), the identification and cultivation of a totally new *Bartonella* species was possible, named after the locality of the sampled bat: *Bartonella naantaliensis* Veikkolainen & Vesterinen 2014 (IV)**.** The new *Bartonella* species did not seem to be closely related to *Candidatus* Bartonella mayotimonensis (IV). Moreover, the same bacteria were found from both the bats and the ectoparasites. This finding confirms the hidden but anticipated interactions in the studied host-parasite network (IV).

4.5. Evaluation of molecular methods in food web research

Biology as a science has come a long way from collecting whole animal specimens in exotic lands to collecting non-invasive samples. In the ideal scenario, the study animal is not even captured or encountered, but instead a trace of the animal (dropping, hair, etc.) is examined and used for detailed analysis. Sounding a lot like science fiction, this is now possible due to the major advancements reached in the field of molecular ecology. The most influential methodological advancement to unravel ecological interactions has been the utilisation of DNA-based approaches (Kaartinen *et al.* 2010). For instance, these molecular methods have allowed researchers to increase accuracy in host-parasitoid networks: one morphologically determined parasitoid species was found to consist of several distinct species based on DNA barcoding (Smith *et al.* 2011). Secondly, molecular approach reveals accurate dietary interactions for ecologically covert animals, such as bats and heir prey (Bohmann *et al.* 2011; Krüger *et al.* 2014a), or predators that liquefy their prey, such as spiders (Piñol *et al.* 2014). Thirdly, DNA-based methods usually offer the only practical way to study endosymbionts and microorganisms (Castro *et al.* 2015; Toomer *et al.* 2015). Indeed, new methods are changing the way we perceive the world although there still are points to be proven and advancements to be taken.

Concern has been raised about the ability of PCR primers used in metabarcoding studies to sufficiently and evenly amplify all the target taxa (Deagle *et al.* 2014). This problem, known as PCR bias, means the property of PCR primers to amplify certain taxa more than others, or that some taxa may not be amplified at all. Similarly, if some prey taxa are ignored or poorly amplifying due to mismatching primers (false negative), the ecological interpretations may be skewed or even wrong. On the other hand, sometimes taxa not eaten may be present in the final prey species list for example due to platform-related sequencing errors (false positive). False negative may be a problem, if the purpose is to study the diet as a whole, that is, to map all prey species no matter how rarely consumed. One drawback when using molecular is, that they cannot directly distinguish whether the prey was captured as juvenile or adult (life stage inaccuracy). On the other hand, it is possible (although not always easy) to pinpoint second predation using information on phenology, parasite-host data, or prior knowledge of species ecology.

The accuracy and diversity of different NGS (also known as High-Throughput-Sequencing; HTS) platforms have been discussed (Metzker 2010; Moorthie, Mattocks & Wright 2011; Bragg *et al.* 2013). However, personally, I feel that the choice of platform is usually based on availability, costs, and scale of the study rather than optimality for a certain study. Another, more ecological, problem is that some prey objects amplified

might actually originate from the gut of a lower level predator (Sheppard et al. 2005) or a parasitoid may be sequenced while inside its host (second predation, sometimes also referred as hyperpredation). Second predation might produce imprecise results if this is not being identified. It has been discussed, however, that low quantities of secondary prey DNA should not produce a major error source when using degraded DNA, such as faecal DNA (Boyer et al. 2015). On a larger scale, when considering all the food web interactions, secondary predation might play a role in population dynamics of some prey taxa. To conclude, molecular detection of animals and plants are revolutionising the field of ecology and conservation (Shokralla *et al.* 2014, 2015; Gibson *et al.* 2014).

Molecular analyses are capable of producing large data sets cost-effectively compared to morphological analysis, for example in the case of bulk insect trap samples (Gibson *et al.* 2014). One major challenge for molecular methods at the moment is the lack of validated quantitative results (King *et al.* 2008; Pompanon *et al.* 2011; Clare 2014). The interpretation of results for many dietary studies might be totally different if a method that allows using read counts could be used (Fig. 7.).

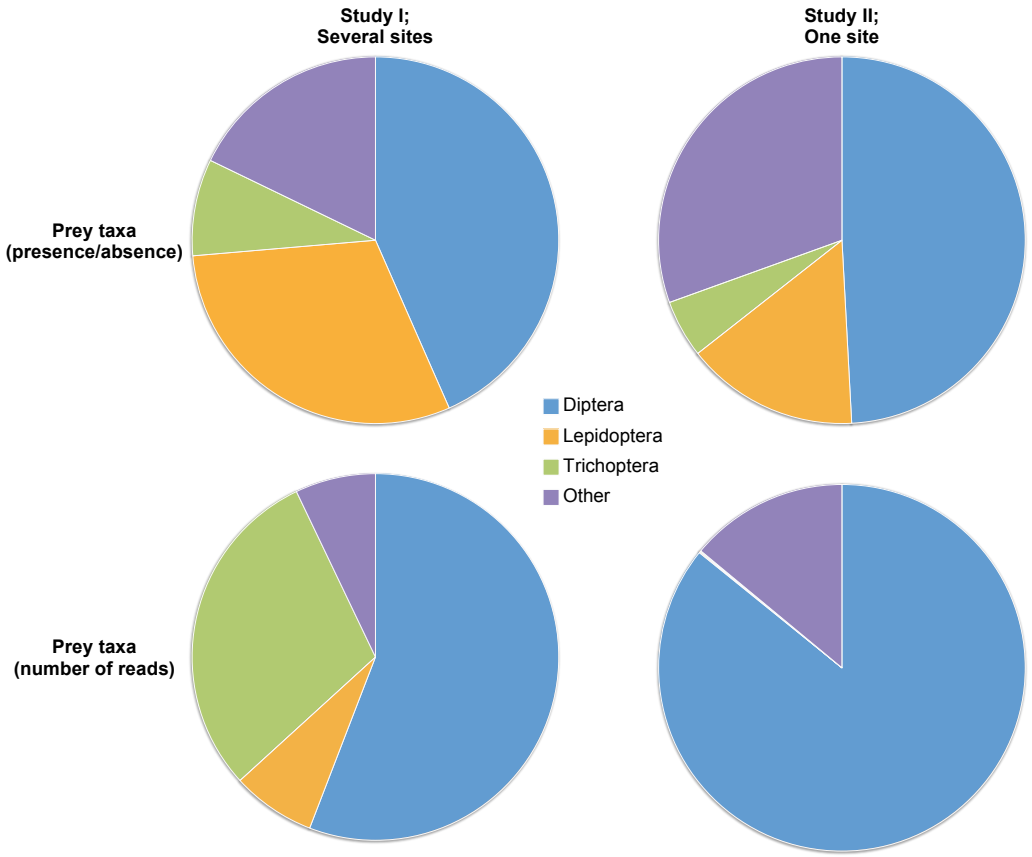

Figure 7. Comparison of the results between frequency data and read count data on the diet of *Myotis daubentonii*. Study I included several locations over southwestern Finland. Study II was carried out in one single location, so there is no habitat-related variation. The proportion of reads assigned to Lepidoptera and Trichoptera in study II (lower right corner) is very small.

There are multiple strategies to reveal the actual proportions of each prey species in a predator's diet. Quantitative PCR (qPCR), or real-time PCR, is one of the most promising methods to reveal the proportions of target DNA. Deagle & Tollit (2007) and Bowles *et al.* (2011) used qPCR to accurately analyse the diet of sea lions with only fairly small error margins between the biomass consumed and prey proportions revealed by qPCR. Despite these promising results, qPCR alone is not practically applicable to generalist predators whose diet may consists of dozens of prey species (see for example Clare *et al.* 2014a; Krüger *et al.* 2014a), since each prey species would need a primer pair of its own which would make the study inefficient and costly. If qPCR is to be replaced with NGS read count data, the method needs to be validated via feeding trials (Deagle, Kirkwood & Jarman 2009).

4.6. Is the molecular approach better than traditional?

To discuss the question, whether molecular approach is good at all, we need to compare it with earlier work. It is a natural phenomenon that dietary ecology varies between species, for which reason the comparison should be restricted to same species between studies. Unfortunately, the lack of studies using different approaches for predators makes comparison practically impossible. Luckily, *Myotis daubentonii* is among the most studied bat species in the world, perhaps due to its vast distribution and large population size (Stubbe *et al.* 2008). Thus, I will mainly focus on *M. daubentonii* to compare traditional and more recent methods to reveal dietary ecology of this nocturnal predator (for a more thorough comparison of the methods, the reader is referred to Razgour *et al.* 2011).

Before DNA-based methods, most dietary studies were conducted through observation of foraging bats (Poulton 1929), examining non-eaten prey remains (Nyholm 1965), by analysis of stomach contents (Kurskov 1968, 1981) or by morphological analysis of insect fragments in droppings (Brosset & Delamare Deboutteville 1966; Swift & Racey 1983; Taake 1991; Sullivan *et al.* 1993; Beck 1994; Flavin *et al.* 2001; Krüger *et al.* 2012; Nissen *et al.* 2013)"plainCitation":"(Brosset & Delamare Deboutteville 1966; Swift & Racey 1983; Taake 1991; Sullivan et al. 1993; Beck 1994; Flavin et al. 2001; Krüger et al. 2012; Nissen et al. 2013. The very beginning of molecular studies was not very promising, since it was very costly and time-consuming to produce even the smallest amount of DNA sequence. Sequencing of a sample containing multiple overlapping targets preceded a laborious cloning to separate different target fragments (Zeale *et al.* 2011). The study by Zeale *et al.* (2011), however, serves as the cornerstone of the molecular research on bat diet, due to the arthropod-specific PCR primers (ZBJ-ArtF1c and ZBJ-ArtR2c) published in that study.

The most striking difference between traditional and molecular methods is the prey identification. Practically all identifications based on traditional methods are limited to order (or equivalent) level (Brosset & Delamare Deboutteville 1966; Swift & Racey 1983; Taake 1991; Sullivan *et al.* 1993; Beck 1994; Flavin *et al.* 2001; Krüger *et al.* 2012; Nissen *et al.* 2013). Contrastingly, the few published studies about *M. daubentonii* diet based on molecular analysis reveal nearly all prey objects to the species level (Krüger *et*

al. 2014a; I, II). For other bat species, there are only a few paired studies allowing direct comparison between morphological and molecular approach. These studies indicate similar results on overall dietary composition as revealed by parallel methods (Razgour *et al.* 2011).

4.7. Biodiversity and human health

All this research, all the funding spent and what is gained? Often this is the only response from the laymen, after they read the results of basic research in a newspaper. Besides, at the time of national and worldwide economic crisis (Caporaso *et al.* 2015), the science organizations need to fight for continuously shrinking funding. Although the public opinion as such is not the driving force for scientists, these questions cry for some justification for scientific research.

As the acquisition of knowledge is important and valuable in itself, there are many aspects linking basic research to benefits to humankind. There has long been a debate whether natural biodiversity is linked to human health. Recently, modern molecular methods have made it possible to rapidly map biodiversity from microhabitats (IV) or from larger bulk samples (Zhou *et al.* 2013; II), thus making it possible to answer the question concerning public health. Undeniably, according to novel discoveries, a decrease in biodiversity is strongly correlated with common illnesses, such as hay fever and allergy (Hanski *et al.* 2012; Haahtela, von Hertzen & Hanski 2013). Moreover, gut macrobiota has been linked to brain functions and even depression in humans very recently (Dash *et al.* 2015). These findings alone highlight the importance of mapping and reporting all kinds of biodiversity, both microbial and macrobial around the whole planet.

5. CONCLUSIONS AND FUTURE DIRECTIONS

There are roughly 1.5 million species already described on the globe and it has been estimated that a vast majority of species are still unknown. In any case, even with the currently known taxa the possible number of interactions among species exceeds our comprehension. Even in the High Arctic where the species number is substantially lower than in the tropical areas, the interaction network is far more complex than one could imagine. This kind of big picture study ought to be expanded to areas of higher diversity to truly map the ecological framework on the planet.

Besides larger organisms, our planet is home for an incredible number of microscopic life forms. While the species number of prokaryotic and viral life forms cannot easily be determined, their abundance is extremely high; the sea floor is inhabited by 290 thousand trillion trillion microbes and soil is estimated to be home for nearly double that amount. Human body alone carries a tenfold of microbial cells compared to our own human cells. Despite these facts, the microbiota is often neglected when constructing interaction networks.

In the light of these findings, it is evident that we still know too little of what is happening around us in nature. We may not yet have a *tricorder* device to answer our questions and truly, I am not sure whether we could comprehend the answer provided by such a device. On the other hand, major advances in technology, breakthroughs in engineering, and new discoveries shed perhaps brighter light on the future of the planet. I call for future studies combining micro and macro scales and expanding the view of life wider than usually thought.

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