University of South Bohemia in České Budějovice

Faculty of Fisheries and Protection of Waters

Research Institute of Fish Culture and Hydrobiology

Bachelor thesis

From the faeces to the food of the invasive species (Molecular diet analysis of the signal crayfish)

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Declaration

I declare that I am the author of this qualification thesis and that in writing it, I have used the sources and literature displayed in the list of used sources only.

České Budějovice, 3. 5. 2024

Jan Škrabánek

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ZADÁNÍ BAKALÁŘSKÉ PRÁCE

(projektu, uměleckého díla, uměleckého výkonu)

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Zásady pro vypracování

Cílem předkládané práce je pomocí molekulárních metod analyzovat potravu raka signálního v Křesánovském potoce vlévajícím se ve Vimperku do řeky Volyňky. Morfologická determinace potravy organismů je povětšinou spjatá s jejich usmrcením, ovšem molekulární metody mohou neinvazivní formou odhalit složení potravy daného organismu.

Úkolem studenta bude především vytvořit přehlednou literární rešerši na dané téma, aby se seznámil s problematikou, a dále pak prakticky pomocí existujících specifických márkrů detekovat možné složení potravy raka z fekálních pelet. Hlavní činností studenta bude kromě analýzy dostupné literatury především práce v molekulární laboratoři, kde bude extrahovat DNA z fekálních pelet a následně připravovat a optimalizovat podmínky PCR reakce se skupinově specifickými márkry. Vyhodnocení bude probíhat metodou zobrazení amplifikovaných úseků DNA v agarózovém gelu, kdy přítomnost či nepřítomnost proužku bude znamenat přítomnost/nepřítomnost DNA dané potravní složky ve fekální peletě.

V průběhu práce student získá zkušenost a návyky k základním pravidlům práce v molekulární laboratoři a také si osvojí použití základních molekulárních technik. Zároveň by se měl naučit orientovat se v odborné literatuře k danému tématu, naučit se ji vyhledávat a získat tak širší povědomí o použití molekulárních metod při analýze potravního spektra organismů, v tomto případě nepůvodního druhu raka.

Rozsah pracovní zprávy:	**25-50 stran**
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Content

1. Introduction

Invasive species are recognized as a significant threat to ecosystems worldwide, with their ability to disrupt delicate ecological relationships and outcompete native organisms. The introduction of non-native crayfish species, in particular, has raised concerns due to their profound impact on native communities. Non-native crayfish, such as the signal crayfish (*Pacifastacus leniusculus*), have become a focal point in invasion ecology. Their rapid colonization of new habitats often leads to substantial changes in local ecosystems. These changes negatively impacts various ecological processes, including nutrient cycling, habitat structures, and predator-prey dynamics. Understanding the ecological consequences of signal crayfish invasion requires an examination of their interactions with native organisms. One crucial aspect of this interaction is the dietary preferences of signal crayfish and how they influence the composition and dynamics of native species communities, especially macroinvertebrate communities.

In this thesis, we applied molecular methods to examine the dietary preferences of signal crayfish and their effects on native communities in the small mountain brook Křesánovský brook, where the invasive signal crayfish has established a strong population. Despite the dense signal crayfish population, the community of macroinvertebrates displayed high diversity and biomass.

Metabarcoding is a common method for determining the diet; however, this method is costly and requires strong reliance on bioinformatic processing of sequencing data. Therefore, besides analyzing the diet preferences of signal crayfish, one of the main aims of this thesis is to find suitable markers for use with classical PCR and electrophoresis. The markers we used in this thesis are already known and applied in the dietary study of other aquatic organisms.

2. Literature review

2.1 Biological invasions

Biological invasions are a worldwide concern that poses a significant threat to ecosystem services and biodiversity (Didham et al., 2005; Diagne et al., 2021). By definition, "invasive species" means an alien species whose introduction does or is likely to cause economic or environmental harm or harm to human health (Exec. Order No. 13112, 1999). The term "biological invasion" described by Valéry et al. (2008) is as follows: *Biological invasion consists of a species acquiring a competitive advantage following the disappearance of natural obstacles to its proliferation, allowing it to spread rapidly and conquer novel areas within the recipient ecosystems where it becomes a dominant population*.

The impacts of invasive species on ecosystems' functioning and native species are severe and, indeed, have sparked debates in academia, politics, and the public (Pimentel et al., 2000). Besides negative ecological impacts, biological invasions cost billions of dollars (Marbuah et al., 2014; Hoffmann and Broadhurst, 2016; Keller et al., 2011). The total costs of invasive species in Europe impacting sectors such as agriculture, forestry, and fisheries calculated between 1960 and 2020 account for 140.20 billion US dollars (116.61 billion euros; Haubrock et al., 2021).

One of the adverse effects of biological invasion on ecosystems is the degradation of the environment and alterations in abiotic conditions, resulting in, for example, native habitat losses (Hermoso et al., 2011; Pyšek and Richardson, 2010). Non-indigenous species are often generalists and, through competition with native species, modify the environment, which makes it difficult for native organisms to inhabit it (Lodge et al., 2000). The cumulative impact of habitat loss often manifests as a simplification of ecosystems and a reduction in species diversity (Miller et al., 1983; Muller et al., 2004). Non-indigenous species outcompete native species due to their ability to adapt to various conditions (Parker et al., 2013) and their better use of available resources (Drenovsky et al., 2012; Ricciardi et al., 2012). This usually leads to biodiversity loss, which typically has adverse consequences for overall ecosystem functioning and the organisms reliant upon it. Mitigation of the impacts of invasive species, therefore, remains a critical priority in protecting the ecological integrity of ecosystems worldwide (Gallardo et al., 2016; Pimentel et al., 2013).

2.2 Aquatic invasions and their spread

The spread and introduction of non-indigenous fishes, aquatic invertebrates, microbes and plants have substantial negative impacts on the functioning of ponds, lakes, streams or rivers worldwide (e.g., Gherardi, 2006; Kajgrová et al., 2022). Non-indigenous species have dominated aquatic environments to a greater extent than their terrestrial counterparts (Vitousek et al., 1997) because aquatic environments are more invasible, more biodiverse, and more at risk of ecosystem-wide changes (Moorhouse and Macdonald, 2015).

Non-indigenous aquatic species are spread and introduced beyond their native environment through various means – one of the main vectors has been international trade and transport by ships (Keller et al., 2011; Wan et al., 2021). Global trade has gradually increased for most of the last 200 years as economies have grown. Nonetheless, the last 50 years have been the most tremendous regarding the importance and value of the merchandise trade (Hulme, 2009). Increased trade has resulted in a legacy of recent biological invasions seen worldwide (Hulme, 2009). Tourism also plays a significant role in the spread of biological invasion (Anderson et al., 2015; McNeill et al., 2011). For example, recreational boats have been a major vector for the spread of the zebra mussel *Dreissena polymorpha* and non-indigenous macrophytes between lakes and rivers within Europe, the USA and New Zealand (Minchin et al., 2003; Rothlisberger et al., 2010).

Moreover, many studies have proven that climate change could further enhance the spread and, consequently, the negative effects of invasions on ecosystems and native communities (Hellmann et al., 2008; Rahel and Olden, 2008; Capinha et al., 2013; Pacheco et al., 2021). Due to global warming, there are predictions that some countries will become more prone to invasion, some will reduce invasion risk by decreasing invasive potentials, and some invaded areas will retreat as they will no longer be climatically suitable for some invasion species. One such example is the spread of *Procambarus clarkii* in Europe – due to global warming, this "warm water" crayfish could occupy previously unavailable or unsuitable habitats (Capinha et al., 2012; Capinha et al., 2013).

While human activities undoubtedly play a significant role in introducing invasive species elsewhere, natural processes could also contribute to the spread of biological invasions (Muthukrishnan et al., 2015). One such example could be presented by the dispersal of invasive aquatic organisms by waterbirds across different regions within a

country (Reynolds et al., 2015; Lovas-Kiss et al., 2024). In running water, the common mechanism of invasion dispersal is upstream migration (Leuven et al., 2009).

2.3 Non-indigenous crayfish in Europe

An increasing number of non-indigenous species have been and continue to be introduced to the European continent (see Stebbing, 2016). Initially, non-indigenous crayfish introductions were primarily driven by commercial purposes such as aquaculture. However, in recent years, most introductions have been due to accidental escapes or deliberate releases of crayfish by pet keepers (Kozák et al., 2015).

Non-indigenous crayfish species (further referred to as NICS) now outnumber indigenous species (further referred to as ICS). In total, ten NICS were introduced in Europe, and at least nine have established populations here. Based on the year of the introduction, we can divide those species into two following groups: the "Old NICS" introduced before 1975 and the "New NICS" introduced after 1980 (Holdich et al., 2009).

The group of the "Old" NICS consists of three crayfish species. All of them originate from North America and are considered the most widely distributed, namely *Pacifastacus leniusculus*, *Faxonius limosus*, and *Procambarus clarkii*. The group of the "New NICS" consists of *F. immunis*, *F. juvenilis*, *F. virilis*, *P. acutus* and *Procambarus* sp. from North America, as well as the Australian species *Cherax destructor* and *C. quadricarinatus*. These introductions generally have more limited distribution in European conditions (Holdich et al., 2009).

One of the biggest threats brought by some of the NICS is the crayfish plague pathogen (*Aphanomyces astaci)*. This pathogen originates from North America, and crayfish native there have adapted to it during many years of evolution – they create a layer of melanin around the growing pathogen and thus prevent further penetration of the pathogen into their bodies (Söderhäll et al., 1979). However, European crayfish species have still not adapted to this pathogen; therefore, up to 100% mortality is reported in infected individuals (Unestam, 1969). Crayfish plague has even been listed among the worst 100 invasive organisms in the world due to its devastating impacts on the populations of European crayfish (Lowe et al., 2000). However, several studies have recently reported that some individuals of European crayfish species (e.g., white-clawed crayfish *Autropotamobius pallipes)* demonstrated some level of resistance to crayfish plague pathogen (Jussila et al., 2015; Martín-Torrijos et al., 2017; Martínez-Ríos et al., 2022).

2.4 Signal crayfish (*Pacifastacus leunisculus)*

Signal crayfish has been considered the most successful crayfish invader, particularly the most widespread NICS, due to its plasticity in both diet and environmental conditions, rapid growth, high fecundity, aggression, burrowing and dispersal ability (Holdich et al., 2014; Kouba et al., 2015; Hudina et al., 2017; Svoboda et al., 2017; Dragičević et al., 2020).

The signal crayfish originating from North America was first introduced to Europe from California in 1959. The first import was into Sweden, which included only around 60 imported individuals (Svärdson, 1995). In 1980, signal crayfish were experimentally also released into Czechoslovakia (Policar and Kozák, 2000). It has spread to most European countries through further introductions directly from America or from recently established European populations (Kouba et al., 2015). More recently, the presence of signal crayfish has also been confirmed in Serbia (Horvatovic et al., 2022).

In its native range, the signal crayfish inhabits various habitats ranging from large rivers to small streams and natural lakes, including submontane lakes. In Europe, it occupies habitats similar to native crayfish species and thrives well also in ponds (Lowery and Holdich, 1988; Holdich et al., 2006).

Surprisingly, the signal crayfish has been found to show relatively low locomotor activity (just under 200 meters per day). This is significantly less than observed in other crayfish species studied worldwide. For example, the narrow-clawed crayfish *Pontastacus leptodactylus* can travel distances even eight times greater in a day, and even the second slowest crayfish species, the noble crayfish, is twice as active as the signal crayfish (Lozán, 2000). Migration speed also appears to be relatively low, both upstream and downstream. Adults migrate downstream on average about 1.5 km per year with a maximum of 13 meters per day, which is common during the summer months. On the other hand, upstream migration tends to be much shorter, depending on the slope of the surface and, therefore, the speed of the current stream. The primary factor, nonetheless, influencing locomotor activity seems to be water temperature (Bubb et al., 2004).

The signal crayfish is a burrowing species of crayfish that excavates burrows in suitable substrate, reaching depths up to 65 cm. By its strong burrowing activity, signal crayfish compensate for the lack of natural shelters in areas where sufficient shelters are otherwise lacking. Nonetheless, such behaviour negatively contributes significantly to bank erosion, especially at high densities of signal crayfish (Guan, 1994).

2.5 Dietary requirements

The signal crayfish is an omnivorous species that feeds on both plant and animal matter. In the early stages of life, the diet of signal crayfish consists mainly of zooplankton, algae, detritus, and zoobenthos. Preference for plant-based components prevails in juveniles, accounting for up to 90 % of the consumed diet, while in adults, it is around 50 %. Food preferences of signal crayfish adjust to the most available food sources during the season (Bondar et al., 2005). Among adult crayfish, there is also reported intraspecific cannibalism (Harlioğlu, 1996; Lozán, 2000). Investigations of the gut content of the signal crayfish revealed a total of 22 different dietary groups during four seasons, including vascular detritus, green algae *Cladophora*, fish, crayfish fragments, insects (Chironomidae, Ephemeroptera, Trichoptera, Coleoptera, Plecoptera, Mollusca, Diptera, *Asellus*, *Gammarus*, Odonata, Oligochaeta, water mites Hydrachnidia, insect eggs and Thysanoptera (Guan and Wiles, 1998; Ercoli et al., 2021; Stenroth et al., 2006).

2.6 The negative impacts of crayfish on ecosystem functioning

Decapods are extremely successful and disruptive invaders in various aquatic ecosystems (Karatayev et al., 2009) with very strong potential to negatively influence ecosystem functioning, mainly by their predation on macroinvertebrates and plant consumption (Jackson et al., 2014; Mathers et al., 2016; Mathers et al., 2020). Crayfish are omnivores and often the largest invertebrates in food webs. Omnivory can decouple trophic cascades and alter energy flow; hence, crayfish as invaders have a disproportional impact on food-webs dynamics (Lodge et al., 1994; Nyström et al., 1999).

Burrowing species of crayfish, such as *P. clarkii* or *P. leuniusculus* can also change ecosystems through the extent of burrowing activity (Faller et al., 2016). The burrowing significantly changes the topography of the riverbed (Johnson et al., 2010), increases gravel transport (Johnson et al., 2011) and generates fine sediment sufficient to drive an increase in suspended sediment concentrations (Harvey et al., 2011; Harvey et al., 2014; Mathers et al., 2022). Such activity also causes changes in nutrient flow (Albertson and Allen, 2015).

Furthermore, invasive species of crayfish are regularly capable of outcompeting and replacing native crayfish (Hill and Lodge, 1999; Dunn et al., 2009; Haddaway et al., 2012). Invasive crayfish species like the signal crayfish reach high population densities due to high consumption rates and the ability to spread fast, which is the main reason for their

even stronger negative effects on ecosystems. Even at low population densities, they can lead to decreasing numbers and diversity in benthic invertebrates and macrophytes as well as a shift in species composition (Vaeßen and Hollert, 2015).

A decrease in macroinvertebrate density was observed in locations where non-indigenous crayfish species were introduced compared to those without invasive crayfish (Crawford et al., 2006; Nyström et al., 2001). Signal crayfish has negative impacts not only on macroinvertebrate communities but also on seedlings and adult macrophytes (Nyström and Strand, 1996). For many fish species, macrophytes serve as an important juvenile habitat (Mittelbach, 1981); hence, a decrease in vegetation cover caused by crayfish can lead to low survival of fish, especially in early life stages (Dorn et al., 1999).

Crayfish invasion also has a negative impact on benthic fish (Light, 2005). Benthic fish (e.g., *Cottus gobio* or *Barbatula barbatula*) compete with introduced invasive crayfish for food resources and shelter. The benthic fish are often outcompeted, and therefore, their densities decrease (Guan and Wiles, 1997). However, the competition of crayfish for food resources does not negatively impact only fish but also native crayfish (Magoulick and Piercey, 2016; Pacioglu et al., 2020; Adami-Sampson et al., 2023).

2.7 Methods for assessment of animal diets in ecology

Biologists and ecologists have posed a seemingly simple question for generations: "What does this animal consume?" The examination of the dietary habits of organisms serves as the bedrock for understanding the interactions that shape ecological communities and regulate the flow of energy and nutrients in food webs (McCann, 2007; Kartzinel et al., 2015). Ecologists nowadays have a variety of methods to examine the diet of organisms. These methods include standard visual techniques such as gut, stomach, faecal, or scat content analyses, DNA identification of prey items, organic macromolecules (e.g., fatty acids), and stable isotope analyses of bulk or specific compounds. The main goal of all these methods is to retrieve precise information on the diet; however, some of these could give biased information on the diet components' quality and quantity (Traugott et al., 2013).

2.7.1 Visual methods of diet analysis

In principle, the best way to understand what animals eat is by directly observing it (Stuart-Smith et al., 2013). While watching animals hunt can apply to big land animals and birds (Pineda-Munoz and Alroy, 2014), it is ineffective for species active at night,

rare, or ones living in the aquatic environment (Hyslop, 1980). While direct observation cannot be possible, examining the contents of their stomachs, gut, or faeces provides information on their diet. However, these methods only provide a snapshot of the consumed prey over a short timeframe. Therefore, they do not seem ideal for studying longer-term patterns (Hayden et al., 2014).

Visual examinations of gut content can show whether the organism has consumed specific dietary items. However, when prey items differ significantly in size, these assessments may not accurately show the quantitative contribution of each food item (Nielsen et al., 2018). Dietary composition can be measured in various ways, such as by counting the number of prey items, assessing their wet or dry mass, or visually assigning scores using a scale from 1 to 10 to estimate their relative importance (Hyslop, 1980). While individual metrics can be combined into a composite index to determine the relative importance of different prey items, this solution may not be optimal when sample sizes are limited (Baker et al., 2014).

Visual techniques, on the downside, do not provide relevant information on the diet if the organism (for example, ants) feeds on a liquid diet (Davidson et al., 2004). Furthermore, it's often hard to identify partially digested food items, so the determination to low taxonomic levels could be impaired. Despite these challenges and the need for experts to identify the partially digested prey, visual methods still give us a lot of useful information and are used in many studies (Pazos et al., 2017; Elfidasari et al., 2020; Mishra, 2020). Additionally, visual methods could consistently provide the different life stages of the prey. However, nowadays, molecular methods to analyze the diet of the organism have been applied more and more frequently due to their high precision and specificity.

2.7.2 Diet analysis through stable isotopes

Stable isotopes are a very useful method of diet analysis that provides time- and space-integrated insights into trophic relationships among organisms. This makes it a very important tool in studying food web interactions (Layman et al., 2012). The stable isotope analysis provides information on the whole spectre of the diet, including secondary resources (the previous diet of the prey). A downside of this method is that it does not offer a species-specific identification of the diet. This is why stable isotopes and DNA-based methods are often combined in studies (e.g., Nelson et al., 2017; Whitaker et al., 2019; Šturm et al., 2021).

2.7.3 DNA-based methods of diet analysis

DNA sequencing of gut content or faeces using high throughput techniques has become a widely used method for studying trophic interactions. This popularity is mainly because these molecular methods provide detailed information about what the animal consumed, even if the identifying prey is rare, soft, highly degraded, or consumed in liquid form (Davidson et al., 2004).

While older methods like protein electrophoresis, immunoassays, monoclonal antibodies, and DNA techniques using cloning and targeted gene approaches (Symondson, 2002) are sometimes still used for tracing diets, most of the current methods rely on high throughput DNA sequencing or target PCR using species- or group-specific markers detected by gel electrophoresis.

Most molecular approaches either target a single species within the sample of interest or analyze a broader diversity of taxa in a mixed sample. Both methods are similar to the eDNA analyses (Bohmann et al., 2014), although inhibitors in the digestive tract can pose problems (Schrader et al., 2012). The aim of the broader diversity option is to identify all organisms consumed by the consumer using either targeted PCR or PCR-free approaches. PCR-based approaches typically rely on some common target genes, such as 16s for bacteria (Huber et al., 2007), ITS for fungi (Lindahl et al., 2013), COI for animals (Pompanon et al., 2012), combinations of ITS, rbcl, matK, trnL, etc. for plants (Valentini et al., 2009), or a series of ribosomal genes for higher phylogenetic placement when broader taxonomic targets are expected (Pompanon et al., 2012).

The precision of molecular techniques enables the identification of species and even specific strains. However, it relies on the availability of reference databases like BOLD (Ratnasingham and Hebert, 2007) for COI, SILVA (Pruesse et al., 2007) for bacterial 16s V6 identification, and UNITE (Abarenkov et al., 2010) for ITS identification, as well as general sequence repositories like GenBank (Benson et al., 2012). Although these reference databases are still incomplete, they are expanding rapidly, and utilizing multiple target regions could enhance both the taxonomic coverage and the likelihood of successful identification (Clare, 2014; Pompanon et al., 2012).

Barcoding represents one of the modern molecular methods of diet analysis. By definition, a DNA barcode is one or more short sequences of genes extracted from a standardized part of the genome, which are used to recognize species. The concept of using these short DNA sequences for biological identifications was first proposed by Paul

Hebert and his associates (Hebert et al., 2003; Hebert et al., 2004), aiming for fast and reliable species-level identifications across all life forms, which includes animals, plants, and microorganisms (Kress and Erickson, 2012). The amplification of the wanted DNA sequence also needs specific primers – such primers must be specially designed and made (Dieffenbach et al., 1993). However, barcoding allows the identification of only a single individual; therefore, the field is now transitioning from barcoding individuals to metabarcoding whole communities (Cristescu, 2014).

Metabarcoding extends DNA-based species identification to communities of individuals belonging to many taxa with distinct roles in the ecosystem (Ji et al., 2013). This multispecies identification method uses massive parallel sequencing of bulk samples (total DNA) or potentially degraded DNA from environmental samples (eDNA) for which species identification via barcoding is not practical (Taberlet et al., 2012; Bohman et al., 2014).

Metabarcoding requires next-generation sequencing, and due to the relatively high expense of this technology, first metabarcoding initiatives were seldom duplicated, tended to be descriptive, and primarily concentrated on investigating specific taxonomic categories (Thomas et al., 2012). However, as costs have significantly decreased, it is now possible to implement more suitable experimental methodologies with technical and biological replication and more rigorous statistical analyses (Cristescu, 2014).

The aims of the thesis were to determine the diet of the signal crayfish in Křesánovský brook and to find and test suitable specific markers for the analysis.

3. Material and methods

3.1 Description of the locality

Křesanovský brook is a left tributary of the river Volyňka, flowing through the town of Vimperk (Fig. 1). The monitored section is located upstream from the town, above the small pond, near the Vodník restaurant.

Sampling was conducted downstream to the culvert under road No. 145. The total length of the sampled area was 450 meters, divided into 50-meter segments where crayfish were captured. For simplification, the stream was divided into two morphologically distinct parts. In the lower section (0–200 m), the channel is deeply incised into the surrounding landscape, with predominantly fast-flowing sections and a rocky substrate. The subsequent section (200–450 m) starts above the mouth of an unnamed stream and extends to the step before the road culvert. This section has a higher proportion of slower-flowing sections, with several semi-natural steps along the entire length (used for snowmaking in winter). Below the steps, the bottom is rocky with faster-flowing water, followed by sections with gradual flow and sandy or gravelly substrate with finer sediments.

The Křesanovský brook has established a very abundant population of signal crayfish (*P. leniusculus*). Crayfish catching and macrozoobenthos sampling were conducted at the site regularly each month from April to October. The lower section was inhabited by a lower number of signal crayfish compared to the upper section. Captured crayfish were transported from the site to the laboratory, where detailed measurements and weighing were conducted, along with the collection of samples.

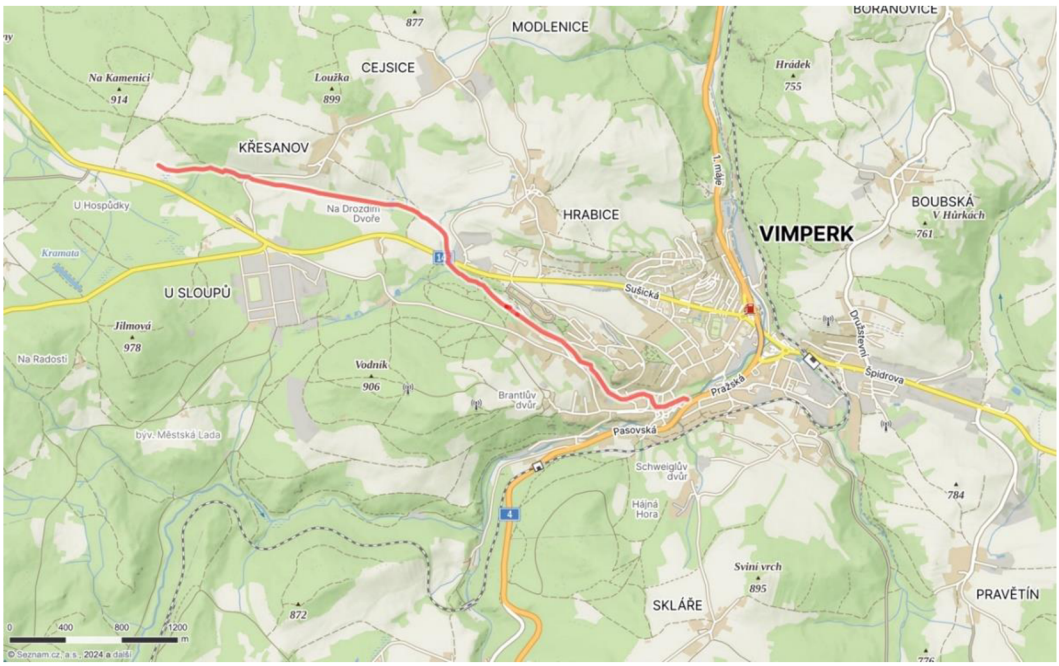

Figure 1: Křesánovský brook shown on the map (red line) (modified from mapy.cz, 2024)

3.2 Collection of the samples

The sampling was done during the year 2020 in months from April to October. Crayfish were sampled from each 50m stretch. Immediately after the crayfish were caught by hand netting, they were washed properly with tap water and individually placed into a plastic container filled with ca 400ml of clean tap water. At least 10 males and 10 females of different sizes were taken. The size and weight of crayfish were recorded after producing and sampling the faecal pellets. Crayfish were kept in plastic containers for different times until they produced faecal pellets. The time usually ranged from several hours to overnight. Faecal pellets were sampled from the plastic container with the dropper and placed into 5ml Eppendorf tubes filled with pure ethanol. The tubes were stored in a fridge until the extraction of DNA.

3.3 Molecular analysis

3.3.1 Extraction of the DNA

DNA was extracted from the faecal pellet using a commercial Nucleospin food DNA kit (Macherey-Nagel). Shortly, 200 mg of the faecal pellet was used and placed into a new Eppendorf 1,5ml tube. Lysis CF buffer and 20 µL Proteinase K were added, and the content was gently mixed. Then, the tube was placed on a thermo-mixer (Eppendorf) and incubated for at least 2 hours at 65°C. Afterwards, the mixture was centrifuged for 10 min (> 10,000 x g), and the clear supernatant was transferred to a new Eppendorf tube. Buffer

C4 and then pure ethanol were added in the same volume as the supernatant, and the whole content was vortexed for the 30s. As a next step, the whole volume was transferred to NucleoSpin® Food Column (NSFC), placed in a Collection Tube, and centrifuged for 1 min at 11,000 x g. The following steps were to wash the DNA caught in the NSFC by washing buffers. Finally, NSFC was centrifuged at a higher speed to remove remains from washing buffers, and then, Elution Buffer was applied to elute the DNA into a new Eppendorf tube. 100µL of Elution Buffer (preheated to 70°C) was applied on the membrane in NSFC, incubated for 5 min at room temperature and centrifuged for 1 min at 11,000 x g. Eluted DNA was stored in a freezer before being used for PCR.

3.3.2 Testing of suitable markers

Since there is no study dealing with crayfish diet analysis using species- or group-specific markers, these were adopted from studies of Koester et al. (2013), Corse et al. (2010) and Sint et al. (2014). The broad food spectrum ranging from detritus to macroinvertebrates was expected as Křesánovský brook is inhabited by species-rich and numerous Ephemeroptera, Plecoptera and Trichoptera (further in text "EPT") taxa (Šťastná, 2023). The markers for trichopterans, ephemeropterans, plecopterans and others (chironomids and gammarids) were tested (Table 1). This was possible due to the DNA library from the most abundant species of EPT taxa produced by Pejcharová (2024). The markers were tested with five genera of Ephemeroptera (*Baetis*, *Epeorus*, *Ecdyonurus*, *Rhithrogena* and *Hapbrophlebia*), five genera of Plecoptera (*Isoperla*, *Nemoura*, *Protonemura*, *Siphonoperla* and *Leuctra*), and four genera of Trichotera (*Rhyacophila*, *Halesus*, *Goera* and *Hydropsyche*). The PCR protocol from the three mentioned studies was adopted. 0.3 µL forward and reverse primers each, 3.4 µL of PCR H_2O, 1 µL of DNA and 5 µL of PPP master mix (TopBio). Additionally, we tested markers for "Characeae" and "Eudicotyledons" (Corse et al., 2010) which should amplify Willow tree (*Salix* sp.), Hornbeam (*Caprinus* sp.), and Poplar tree (*Populus* sp.), respectively, the trees that growth in surroundings of the brook and therefore should be the basis of coarse detritus. All PCR reactions were conducted on TProfessionalTRIO and T300 Thermocyclers, both from Biometra (Fig. 2).

Figure 2: TProfessionalTRIO and T300 Thermocyclers

For testing primers and optimization of protocols, the DNA originating in the work of Pejcharová (2024) was used. Following primers were applied to cover the most common species present in the brook and possible crayfish food items (Table 1).

Table 1: List of primers used and tested in this study.

Marker name	Amplified species/group	Annealing temperature	Multiplex	Fragment length [bp]	Source
Inspi18Sf	Trichoptera Integripalpia + Spicipalpia	56°C	M1	306	Koester et al. (2013)
Inspi18Sr	Trichoptera Integripalpia + Spicipalpia	56°C	M1	306	Koester et al. (2013)
Epa28Sf	Ephemeroptera	56°C	M1	135	Koester et al. (2013)
Epa28Sr	Ephemeroptera	56°C	M1	135	Koester et al. (2013)
Hpt28Sf	Heptagenidae	56°C	M1	550	Koester et al. (2013)
Hpt28Sr	Heptagenidae	56°C	M1	550	Koester et al. (2013)
Chiro18Sf	Chironomidae	50°C	M2	355	Koester et al. (2013)
Chiro18Sr	Chironomidae	50°C	M2	355	Koester et al. (2013)
Ple-gen A269	Plecoptera	50°C	M2	117	Sint et al. (2014)
Ple-gen S268	Plecoptera	50°C	M2	117	Sint et al. (2014)
Gamae28Sf	Gammaridae	56°C	M3	316	Koester et al. (2013)
Gamae28Sr	Gammaridae	56°C	M3	316	Koester et al. (2013)
CHA677	Characeae	56°C	M3	1022	Corse et al. (2010)
CHA1681R	Characeae	56°C	M3	1022	Corse et al. (2010)
MYR152F	Eudicotyledons	56°C	M3	1570	Corse et al. (2010)
MYR1696R	Eudicotyledons	56°C	M3	1570	Corse et al. (2010)

3.3.2.1 Multiplexing

For the needs of the diet analysis of the signal crayfish from Křesánovský brook, 8 samples from each month were tested on all multiplexes.

Markers were tested in multiplexes based on similar annealing temperatures and different lengths of the amplified fragments (Table 1.). Multiplexes were first tested on the DNA of particular macrozoobenthic species to ensure the reaction was successful with an amplicon of appropriate size. The total volume of PCR reaction was 12.5 μL. The mixture of each multiplex reaction contained 0.3 μL forward and reverse primers from each group, PCR H_2O was added so that it had 5 μL microliters including primers, 1.5 μL of DNA and 6 μL of TEMPase Hot Start 2× master mix (VWR).

In all runs, positive and negative control was used. The DNA of particular representatives of each group that had already been used for testing the primers was used as a positive control. PCR water was used as a negative control instead of the DNA. Afterwards, those controls were used in every PCR run, which was prepared to control the purity of the PCR reaction preparation.

According to amplicon sizes, three multiplexes were established (Table 1). Integripalpia/Spicipalpia (306 bp), Heptagenidae (550 bp) and Ephemeroptera (135 bp) were grouped in Multiplex 1 (M1). Chironomidae (355 bp) and Plecoptera (117 bp) were grouped in Multiplex 2 (M2). Gammaridae (316bp), and primers marking representatives of macrophytes Characeae (1022 bp) and Eudicotyledons, i.e., Salicaceae (*Populus* sp.), Haloragaceae (*Myriophyllum* sp.), Fabales, and Caprifoliaceae (1570 bp) were grouped to Multiplex 3 (M3) (see Table 1.).

After finding out that both multiplex reactions work, all chosen samples of the faecal pellets were tested using the same procedure. The PCR protocols for these multiplex reactions started with initial denaturation at 95°C for 5 minutes, followed by 30 cycles of 95 °C for 40 seconds, annealing temperature 56 °C for 40 seconds and extension at 72 °C for 90 seconds. The final extension was at 72 °C for 10 minutes. Multiplexes 1 and 3 had an annealing temperature 56 °C, while multiplex 2 had a lower annealing temperature of 50°C.

Each preparation of the PCR reaction was conducted in the polystyrene box with ice (Fig. 3).

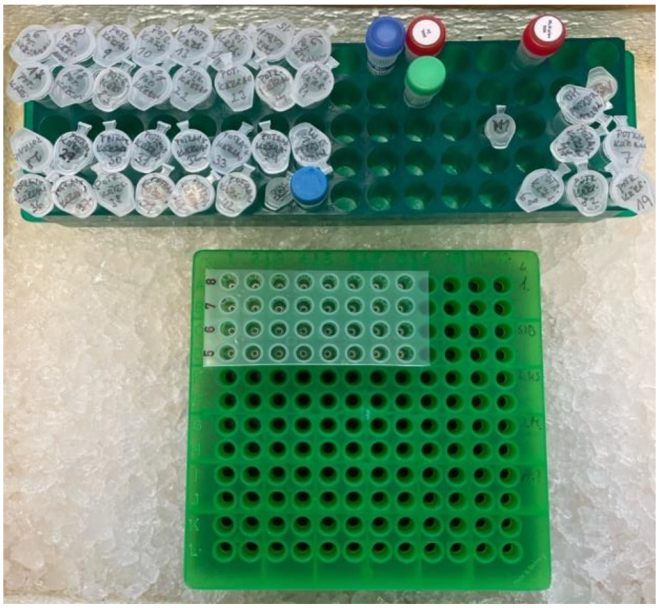

Figure 3: Preparation of the PCR reaction

3.3.3 Gel electrophoresis

To determine the PCR results, gel electrophoresis was the following step. Electrophoresis was conducted on an agarose gel (usually 1,5-2%). For the DNA visualization, 3 µl of Gold View dye was added to the beaker with dissolved agarose. When testing with a single marker and PPP MasterMix, 10 µl of PCR product was loaded into the gel, while with multiplexes and colourless TEMPase master mix, 8 µl of PCR product and 3 µl of loading dye solution were mixed and loaded to wells in the gel. To determine the length of particular PCR products, a DNA ladder (100-1500bp) was loaded.

After the gel electrophoresis ended. The gel with DNA was illuminated with UV light and photography of the bands (Fig. 5) was taken.

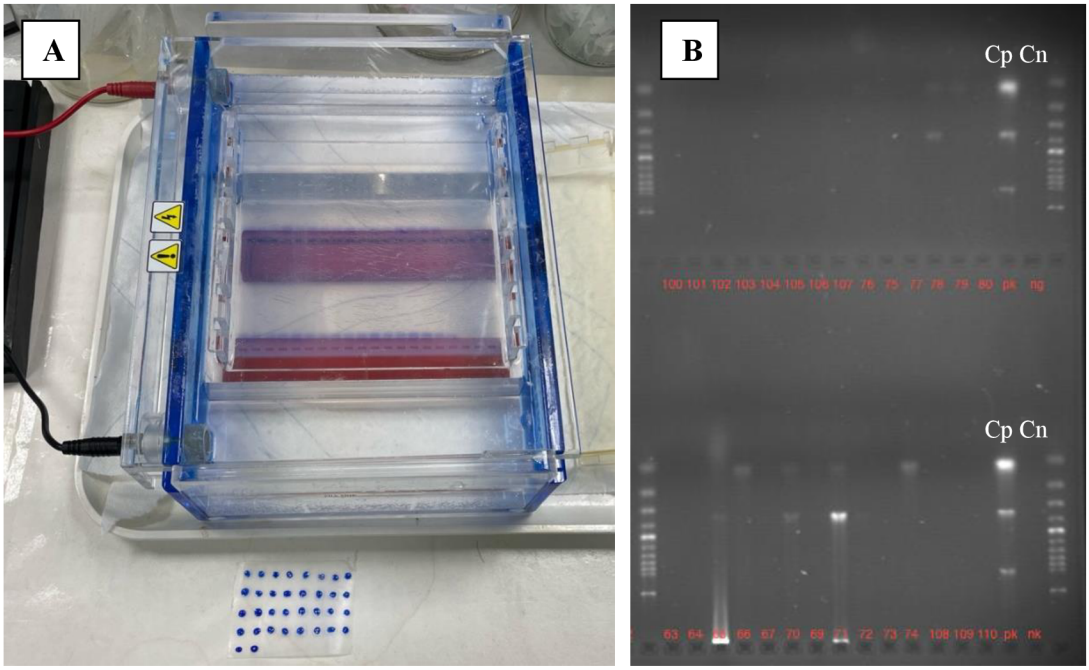

Figure 4: Gel electrophoresis with loading dye drops below (A). Photography of the gel with applied Multiplex 2, the first and the last well is loaded with the DNA ladder, while Cp and Cn means positive and negative control, respectively (B).

3.3.4 Data processing and evaluation

The data from the photos was converted to digital form in Microsoft Office Excel. For each group positive band was marked as "1" and samples without band were marked as "0". In this way, data were obtained on the number of organisms in one sample. The graphs were made in RStudio, with use of the "ggplot2" and "dplyr" libraries. For better visualization, some graphs were adjusted in Adobe Photoshop.

The frequency of occurrence of individual food components was determined. This index was calculated according to Amundsen et al. (1996):

$$Fo = \frac{\mathrm{nF}}{\mathrm{n}} \cdot 100\ [\%]$$

nF – number of samples containing the food component

n – total number of samples

F*o* – this index indicates the % occurrence of a food component during the season for all samples

The trophic overlap index between the sampling months was calculated according to Schoener (1974):

$$D = 1 - 0.5 \sum_{i=1}^{n} |Pxi - Pyi|$$

n – number of the food components

Px, Py – share of the food component in specific month

D – this index shows the trophic overlap between the months

4. Results

4.1 Finding suitable markers and establishing multiplexes

The target groups of macroinvertebrates representing diet items, i.e., markers, were grouped according to similar annealing temperatures and different amplicon sizes. The results of the tests found that Trichopteran Integripalpia/Spicipalpia (Inspi18Sf + Inspi18Sr), Heptagenidae (Hpt28Sf + Hpt28Sr), Ephemeroptera (Epa28Sf + Epa28Sr), Gammaridae (Gamae28Sf + Gamae28Sr), Characeae (CHA677 + CHA1681R) and Eudicotyledons (MYR152F + MYR1696R) could be annealed withing the same annealing temperature of 56 °C, while Chironomidae (Chiro18Sf + Chiro18Sr) and Plecoptera (Ple-genA268+ Ple-genS267) were amplified using annealing temperature 50 °C. Due to the overlapping and similar size of particular amplicons, two multiplexes with an annealing temperature 56 °C were established. The first multiplex (M1) contains trichopteran Integripalpia/Spicipalpia (306 bp), Heptagenidae (550 bp) and Ephemeroptera (135 bp). Chironomidae (355 bp) and Plecoptera (117 bp) were grouped in the second multiplex (M2). Gammaridae (316bp), and primers marking representatives of macrophytes Characeae (1022 bp) and Eudicotyledons (1570 bp) were grouped to third multiplex (M3) (Table 1.).

4.2 Analysis of the diet

In total, during the whole sampling season, 48 samples were tested by target PCR using three multiplexes. The most abundant food item detected in the diet of the signal crayfish was Gammaridae, found in 26 samples of faecal pellets (54,1 %) followed by Heptagenidae along with Integripalpia and Spicipalpia, found in 15 samples of faecal pellets (31,3 %). Plecopterans were found in 10 samples of faecal pellets (20,8 %). The other groups, Chironomidae and Characeae, were each found in 5 samples of faecal pellets (10,4 %). Eudicotyledons were detected in only 4 samples, making 8,3 % abundance in the diet (see Fig 6). Ephemeroptera were the least abundant group, detected only in one sample of the faecal pellets (2,1 %)

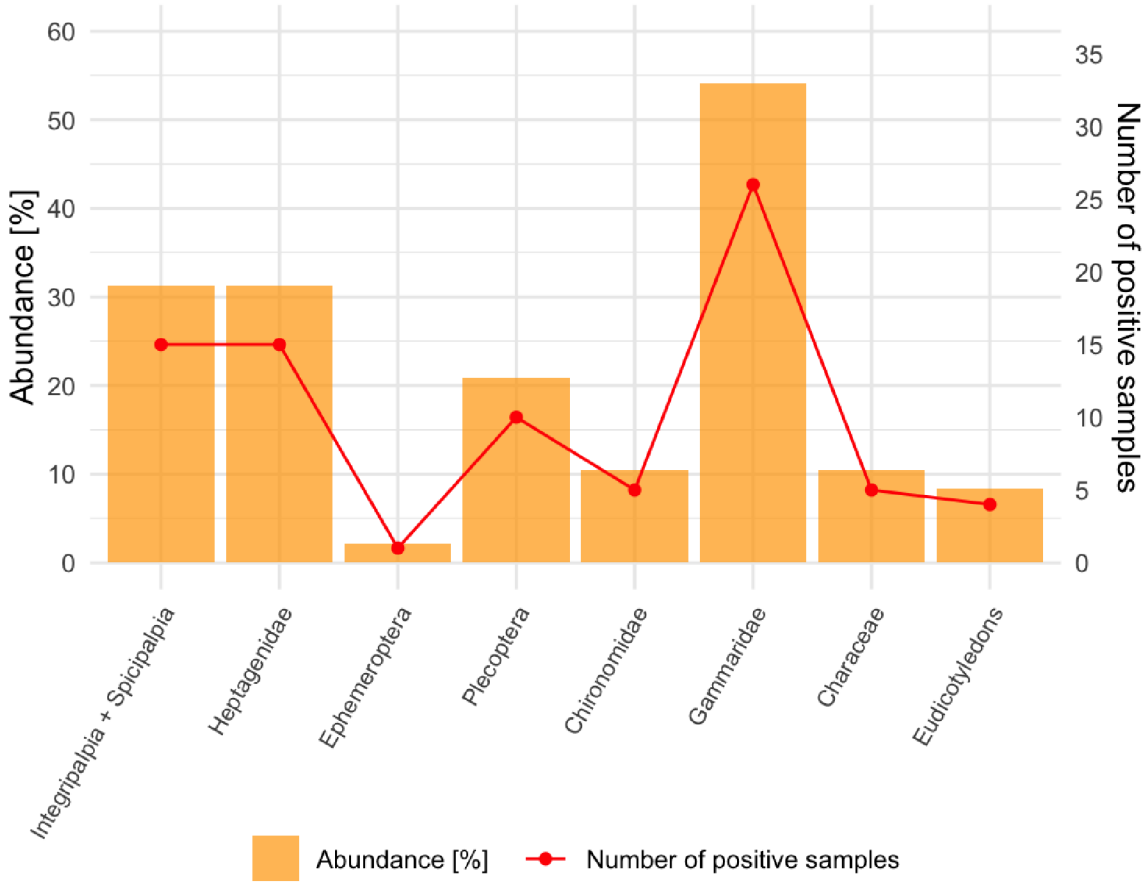

Figure 6: Abundance [%] of the dietary groups during whole sampling season

In samples from April (15.4.), the most abundant group detected in the diet was Gammaridae detected in 8 samples of faecal pellets, followed by Heptagenidae found in 3 samples. Representatives of Plecoptera were found in 2 samples. Eudicotyledons and Characeae were both found in only 1 sample each. However, groups of Integripalpia and Spicipalpia, Ephemeroptera and Chironomidae were not detected in the samples from this date (Fig. 7a).

From samples collected in the middle of May (14.5.), almost twice as many dietary items were found compared to the previous month. The most significant group in the diet was Gammaridae, found in 6 samples. The second most abundant diet group detected was Integripalpia/Spicipalpia (6 samples). Groups of Heptagenidae, Plecoptera and macrophyte Characeae were each found in 3 samples of faecal pellets. The less significant group was macrophyte Eudicotyledons, with only 1 positive sample. Ephemeroptera were not found in any of the samples (Fig. 7b).

Sampling from the first half of June (8.6.) showed that Gammaridae were found in all samples, making it the most significant group from this month. Integripalpia and Spicipalpia were found in a total of 7 samples of faecal pellets. Heptagenidae were visible in 4 samples, as well as groups Plecoptera and Chironomidae. Ephemeroptera, Edicotyledons and Characeae we all found in 1 sample each (Fig. 7c).

Results from July (8.7.) found out, that the most abundant group in this month of the diet was Heptagenidae with 5 positive samples. Gammaridae showed up in 4 samples of faecal pellets. Representatives of Integripalpia and Spicipalpia were found in a total of 3 samples. In only 1 sample group Plecoptera, Chironomidae and Eudicotyledons were positive (Fig. 7d).

From August (18.8.) no amplified DNA bands were visible in any of the samples (Fig. 7e).

Samples from September (15.9.) were almost without bands. In 1 sample, only group Gammaridae was found during this month, in other samples no amplified DNA bands were visible (Fig. 7f).

At the end of the season in October (20.10.) no dietary groups were found in the samples at all (Fig. 7e). The overall results from all months are summarised in Figure 8.

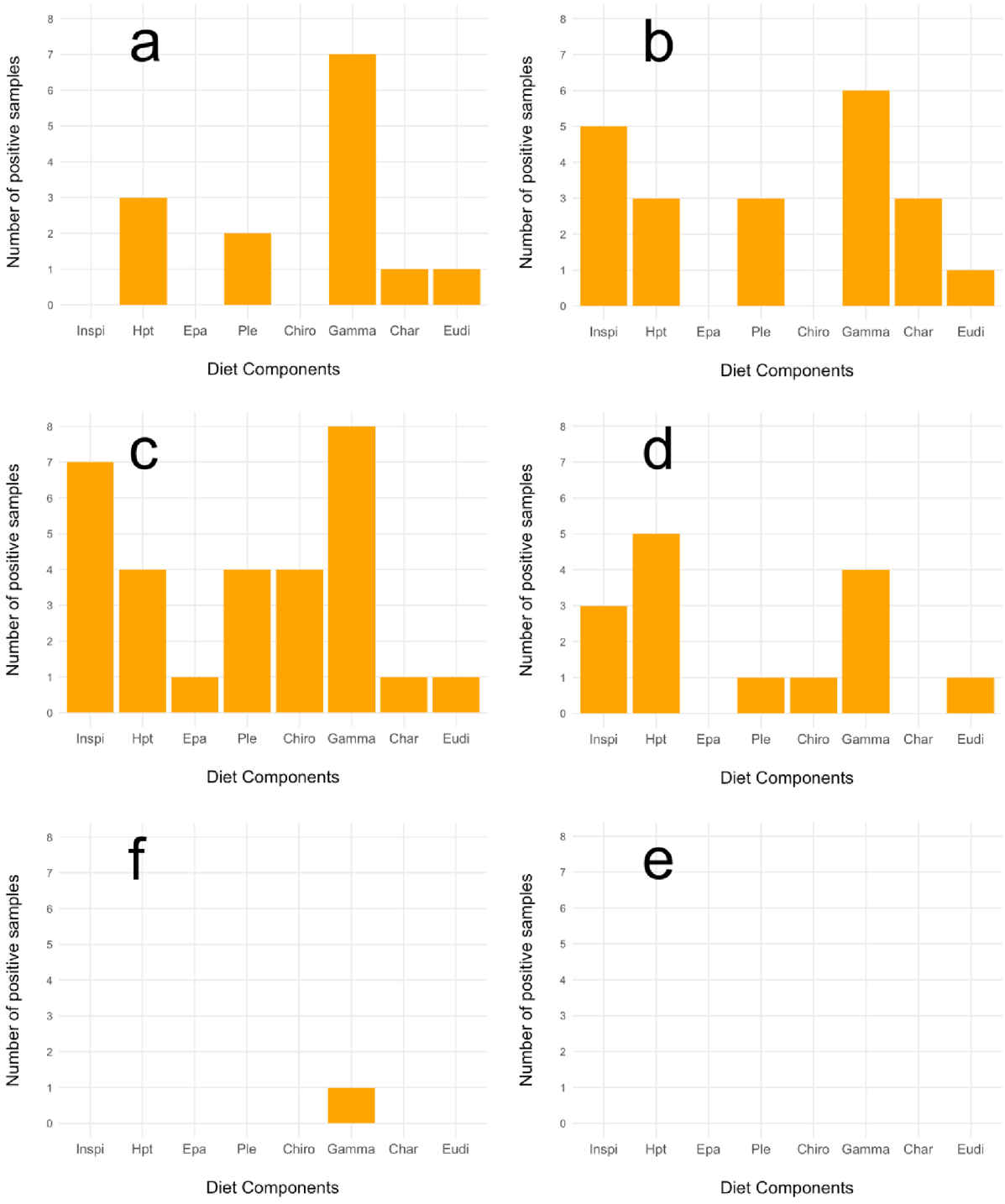

Figure 7a-f: The number of positive samples in different dietary components across the sampling season (a April, b May, c June, d July, e, August/October, f September), (Inspi = Integripalpia +Spicipalpia, Hpt = Heptagenidae, Epa = Ephemeroptera, Ple = Plecoptera, Chiro = Chironomidae, Gamma = Gammaridae, Char = Characeae, Eudi = Eudicotyledons)

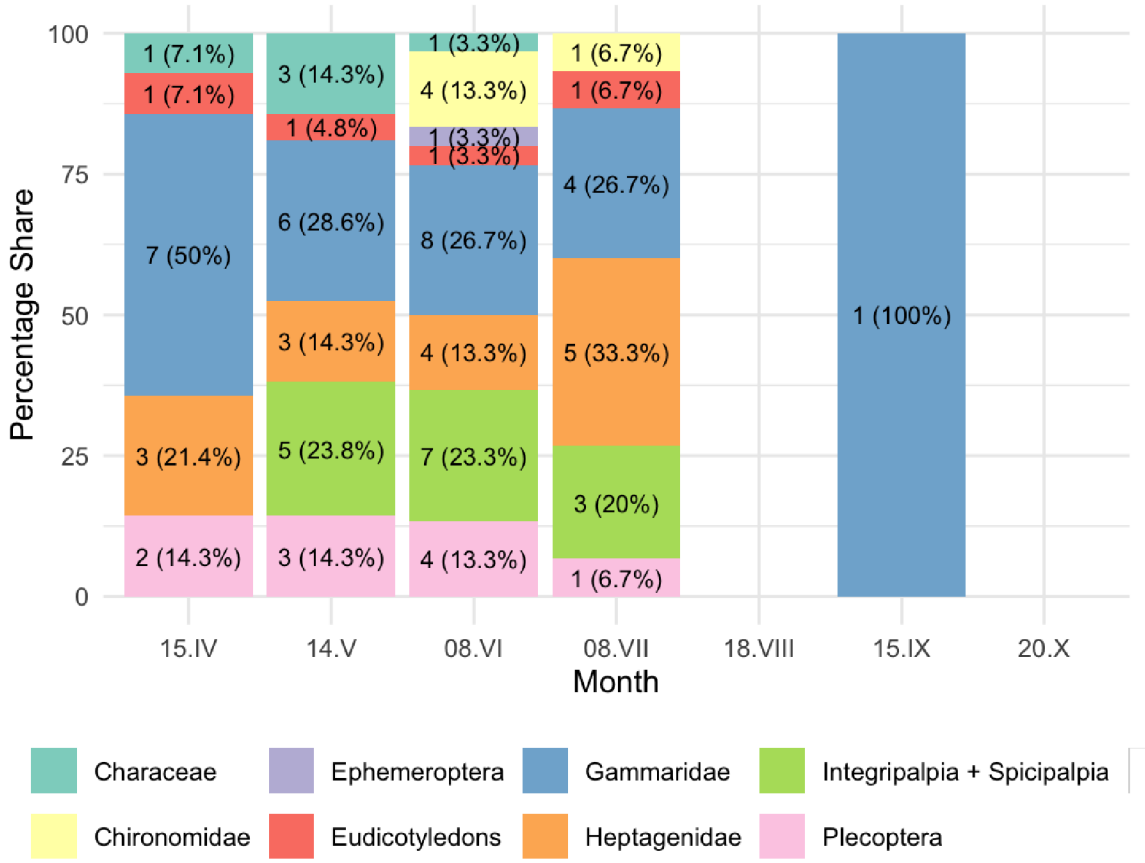

Figure 8: Share of the diet components [%] across months in the number of samples detected - summary

In this thesis, we also calculated Schoener's overlap index to measure how similar resource utilization was among taxa across the sampling season from April to October (Fig. 9). Comparing April to other months, we observed moderate overlap with May, indicating some similarity in resource usage. However, April showed low overlap with the later months, suggesting different ecological patterns. May and June had a high overlap, suggesting a continuity in resource use between the two months. May and July had a moderate overlap, while May had a low overlap with the later months, indicating changes in resource availability. June and July also had high overlap, pointing to similar resource use across these months. However, the overlap between June and August was low, indicating a change in resource usage. July's overlap with August was minimal, suggesting different ecological dynamics. Similarly, overlap with the later months (September and October) was low. August, September, and October all showed low overlap with the earlier months, indicating distinct shifts in resource usage during these months. In summary, May, June, and July had a higher overlap, while April, August,

September, and October showed distinct patterns. This information helps us understand seasonal variations and ecological interactions in the studied taxa (Fig. 9).

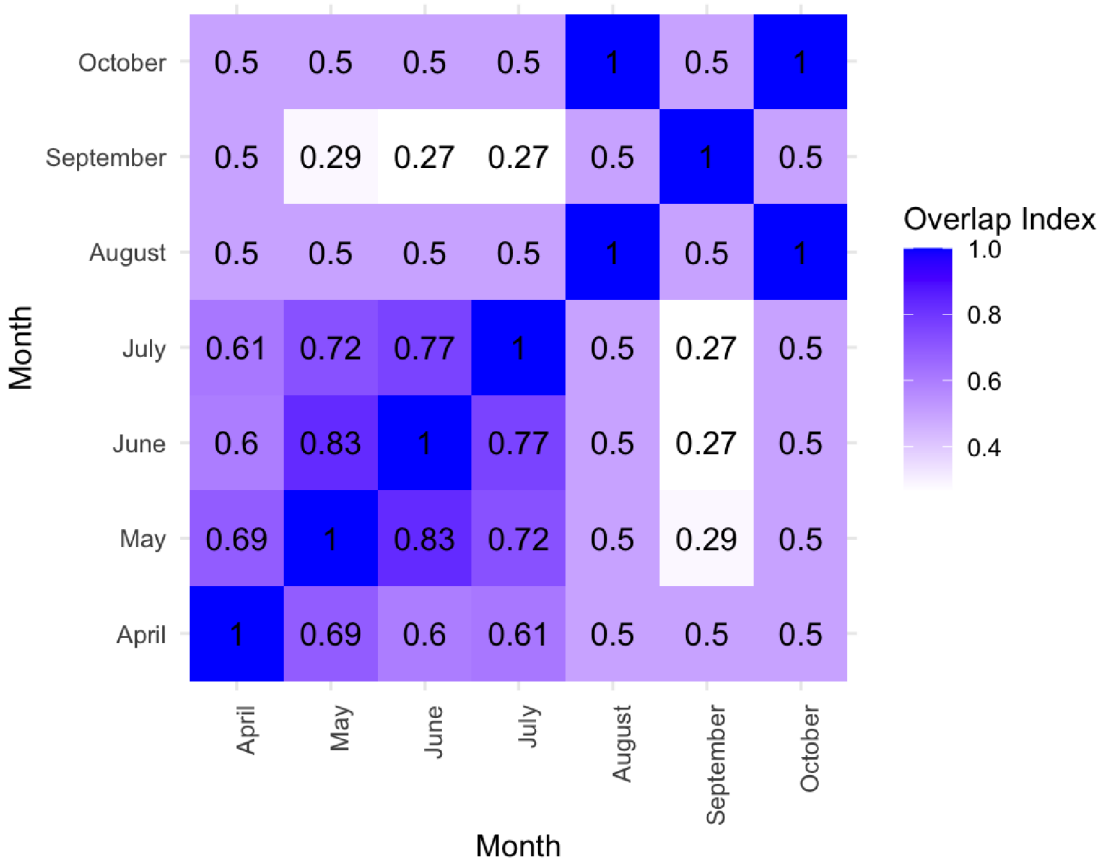

Figure 9: The heatmap of the Schoener's overlap index across different sampling months. The heatmap shows overlap between pairs of months, with values ranging from low (white) to high (blue). We annotated each square with the index value for clarity. The data included various food items and their abundances during sampling season.

5. Discussion

5.1 Determination of the diet

The diet of the signal crayfish from the whole season consisted of macrozoobenthos EPT taxa (Trichopteran Integripalpia/Spicipalpia, Heptagenidae, Ephemeroptera, Plecoptera) and all other detected groups Chironomidae, Gammaridae, Characeae and Eudicotyledons, which has already been confirmed by few studies (Guan and Wiles, 1998; Ercoli et al., 2021; Rosewarne et al., 2016; Stenroth et al., 2006; Bondar et al., 2005). However, research in this thesis is limited to only a few groups because studies mentioned above show that the diet of crayfish contains many more dietary groups than those described in this thesis. All of the mentioned studies analysed the diet of crayfish by direct visual examination of the gut content, which could bring a higher diversity of detected food items. DNA analysis sometimes cannot target the specific DNA, because of its degradation (Deagle et al., 2006), however, under good circumstances, DNA analysis can tell us much more about the diet than visual examination.

Overall, in this thesis, the most abundant group in the diet of the signal crayfish was Gammaridae; however, none of the published research focused on the crayfish diet found this group as the most abundant (e.g., Ercoli et al., 2021; Rosewarne et al., 2016). The most numerous diet items in these studies were detritus and periphyton. Plant-based diet components in our research were represented as Characeae, and their abundance in the diet was low. We could not determine "detritus" as a food component because it is difficult to detect this group containing many sources of DNA as one record (positive bend in gel) by DNA methods, while studies using visual methods allow it. Detritus consists mainly of organic matter, including dead plants and/or animal matter. DNA-based methods allow us to detect the exact components of the detritus, which gives more details about the consumed diet. Rosewarne et al. (2016) and Bondar et al. (2005) determined Chironomidae and Heptagenidae as the most abundant macroinvertebrate groups in the signal crayfish diet. However, it is important to highlight that diet composition depends on the availability of food resources in the habitat of the crayfish. Šťastná (2023) evaluated the composition of the macroinvertebrates in the Křesánovský brook. According to her results, the most abundant macroinvertebrate in this stream was *Rhyacophila sp.* (Heptagenidae), Plecoptera and Amphipoda. This confirms the abundance of these groups in the diet under our study. During the end of the sampling

season, the Gammarids were detected in only one sample of the faecal pellets in September. Overall, the abundance of the whole diet during August, September and October was very low (Fig. 8). This could be caused by the lower feeding activity of the crayfish induced by rapid changes in the temperature, yet we did not find the data of the water temperature in Křesánovský brook during the sampling season.

On the other hand, we could not rule out the negative detection of food items was caused by problems with the extraction of DNA. However, the DNA of the faecal pellets was extracted in a bulk of samples using the exact same procedure. In this extraction run were also samples, for example, from June and July, which indicated the presence of the dietary groups. Hence, there is almost no chance that the DNA was unsuccessfully extracted. The amplification of the DNA is the same case as the extraction. In the PCR runs, more samples from more months, including August, September, and October, were used. Despite the same process of combining primers and other PCR reaction components, many of the samples showed the presence of dietary groups. However, Guan and Wiles (1988) did a visual gut content analysis of the signal crayfish and found many prey items during the whole season (also in winter), which does not fit with the results from this thesis. If we tried to detect more types of food components in the diet of the signal crayfish, such as more macrophyte or even macroinvertebrate groups, we could detect feeding activity even during the end of the season.

Macrophytes were detected in the samples of faecal pellets; however, this group was not as abundant as it is described in recent studies. Another problem with the determination of the macrophytes in the diet was that we did not have a positive control for macrophytes because the control DNA was not isolated. Despite having no positive control for macrophytes, we decided to test it; however, we are aware that this approach is not ideal. Another problem of this research could be the limited dataset. More samples of faecal pellets should be tested each month for a more accurate description of the diet.

5.2 Establishing multiplexes

It is important to ensure that each multiplex contains amplified fragments of food components with distinct lengths to establish multiplexes properly. If the lengths of the amplified fragments were close to each other, we would not be able to recognize it properly, due to the limitation of the agarose gel usage. In that regard, capillary electrophoresis could be the solution to prepare less multiplexe with markers amplifying similar amplicons differing in only several base pairs. Therefore, we made three multiplex

reactions and distributed the food components with the same fragment length to different multiplexes.

However, the annealing temperatures of some of the markers used in this thesis differ in annealing temperatures in original publications (Koester et al., 2013; Corse et al., 2010; Sint et al., 2014). For instance, the annealing temperature of the marker for Gammaridae is 60 °C according to Koester et al., 2013, but we found out that it could be amplified even with an annealing temperature 56 °C. Another example is the annealing temperature of the markers for Plecoptera, which is 60 °C according to Sint et al., 2014. In this thesis, the annealing temperature of this marker was 50 °C. The point is that some of the markers can work within quite a wide range of annealing temperatures, which was here tested by several gradient PCR reactions in which we found amplicons at mentioned temperatures different from the original ones.

6. Conclusion

In this thesis, we brought new insight into the food behaviour of signal crayfish. We used molecular methods to detect particular food items from DNA extracted from faecal pellets taken from representatives of the signal crayfish population in Křesánovský brook from April to October 2020. We also found and tested suitable markers for the detection of food components in the diet of signal crayfish. The diet analysis was performed using the DNA extracted from the faecal pellets taken directly from the signal crayfish representatives in this stream. For the detection of the DNA in faecal pellets, suitable group-specific markers were found in published studies. These markers were tested, used, and described in this thesis. We focused on the macroinvertebrate groups representing EPT taxa, Chironomidae and Gammaridae. Additionally, the representatives of macrophytes were also detected by using specific markers.

Analysis suggests that the crayfish in the Křesánovský brook preferred different types of food items during the sampling season (Apr-Sept). The most common food item was Gammaridae, found in over half of the samples. Other important food groups included Heptagenidae, Integripalpia, and Spicipalpia, which were all present in a third of the samples. Plecoptera were found in over 20 % of the samples. Other groups were less abundant.

Although this study provides insights into the diet of the signal crayfish, there is a possibility to continue the research. In future studies, a broader diversity of the food components could be included in a higher amount of tested samples to provide more accurate data about the diet through the season, including winter.

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8. Abstract

This thesis aimed to determine the diet of the signal crayfish in Křesánovský brook in 2020. In addition to that, we aimed to find and test suitable markers for the diet analysis of signal crayfish, which are described in the thesis. We analyzed the crayfish diet by examining DNA from faecal pellet samples collected monthly from April to September. By using group-specific DNA markers, we targeted the main dietary groups such as Heptagenidae (markers Hpt28Sf + Hpt28Sr), Ephemeroptera (Epa28Sf + Epa28Sr), Integripalpia and Spicipalpia (Inspi18Sf + Inspi18Sr), Plecoptera (Ple-gen A269 + Ple-gen S268), Chironomidae (Chiro18Sf + Chiro18Sr), and Gammaridae (Gamae28Sf + Gamae28Sr). We also focused on representatives of macrophytes Characeae (CHA677 + CHA1681R) and Eudicotyledons (MYR152F + MYR1696R). The results showed that the crayfish's diet varied during the sampling season. Gammaridae was the most common food item, found in 54.1% of the samples of faecal pellets. Heptagenidae, Integripalpia, and Spicipalpia were also significant, present in 31.3% of the samples. Plecoptera appeared in 20.8% of the samples, while Chironomidae and Characeae were found in 10.4%. Eudicotyledons were detected in 8.3% of the samples. Ephemeroptera were the least common food group, detected only in 2.1 %. Gammaridae were the most significant component of the crayfish diet in April (found in 7 samples), while Heptagenidae were the second most abundant component. The diet was more diverse in May, but Gammaridae remained the primary food source, followed by Integripalpia and Spicipalpia (in 5 samples). In June, Gammaridae was present in all samples, while Heptagenidae and Integripalpia/Spicipalpia were significant. In July, Heptagenidae was the most common food group (found in 5 samples), with Gammaridae found in 4 samples. No food items were detected in the samples from August and October, and only one sample contained Gammaridae in September. Overall, this study provides insights into both the seasonal variations in the diet of signal crayfish and markers suitable for its analysis.

Keywords: signal crayfish, diet analysis, DNA markers, group-specific markers

9. Abstrakt

Tato bakalářská práce měla za cíl analyzovat potravu raka signálního v Křesánovském potoce během roku 2020, v měsících od dubna do října. Dalším cílem bylo najít a otestovat vhodné markery pro analýzu potravy. Tyto markery jsou podrobněji popsány v textu této práce. Analyzovali jsme potravu raka signálního pomocí izolované DNA ze vzorků fekálních pelet odebíraných každý měsíc. Pomocí skupinově specifických DNA markerů jsme se zaměřili na klíčové potravní skupiny makrozoobenthosu. Tyto skupiny jsou Heptageniidae (markery Hpt28Sf + Hpt28Sr), Ephemeroptera (Epa28Sf + Epa28Sr), Integripalpia a Spicipalpia (Inspi18Sf + Inspi18Sr), Plecoptera (Ple-gen A269 + Ple-gen S268), Chironomidae (Chiro18Sf + Chiro18Sr) a Gammaridae (Gamae28Sf + Gamae28Sr). V potravě byla také analyzována makrofyta Characeae (CHA677 + CHA1681R) a dvouděložné rostliny (MYR152F + MYR1696R).Výsledky ukázaly, že potrava raka signálního se v průběhu sezóny měnila. Během celé sezóny byly nejčastější potravní skupinou Gammaridae, nalezené v 54,1 % vzorků fekálních pelet. Skupiny Heptageniidae, Integripalpia/Spicipalpia byly také hojně zastoupené (detekovány ve 31,3 % vzorků). Skupina Plecoptera se objevila ve 20,8 % vzorků fekálních pelet, zatímco Chironomidae a Characeae byly nalezeny v 10,4 %. Dvouděložné rostliny byly zastoupeny v 8,3 % vzorků. Nejméně početnou skupinou byly Ephemeroptera, které byla detekována pouze v jednom vzorku fekálních pelet (2,1 %).V dubnu byly Gammaridae nejvýznamnější složkou potravy, a Heptageniidae byli drouhou nejzastoupěnější skupinou.V květnu byla strava pestřejší, ale Gammaridae zůstaly hlavním zdrojem potravy (nalezeny v 6 vzorcích) následované Integripalpia/Spicipalpia (v 5 vzorcích). V červnu byly Gammaridae přítomny ve všech vzorcích, zatímco Heptageniidae a Integripalpia/Spicipalpia byly významné. V červenci byly Heptageniidae nejčastější potravní skupinou (v 5 vzorcích), druhou byly Gammaridae (nalezeny ve 4 vzorcích). V srpnu a říjnu nebyly ve vzorcích nalezeny žádné potravní skupiny, a v září byly Gammaridae přítomni pouze v jednom vzorku fekálních pelet. Celkově tato studie poskytuje přehled o sezónních změnách v potravě raka signálního a markerech určených pro její analýzu.

Klíčová slova: rak signální, analýza potravy, DNA markery, skupinově specifické markery