ECOPHYSIOLOGICAL PERFORMANCE AND LIFE CYCLE STRATEGIES OF NORTH SEA SHRIMPS

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Ecophysiological performance and life cycle strategies of North Sea shrimps

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SUMMARY

The brown shrimp, *Crangon crangon*, inhabits most European coasts with maximum densities in the shallow coastal areas of the southern North Sea. Due to high abundances and reproduction rates, this species is an important member of the coastal ecosystem and a valuable target for local fisheries. The biological success of the brown shrimp in the North Sea indicates that this species is well adapted to a variable environment, in terms of abiotic and biotic factors, where food quantity and quality can change rapidly and severely with seasons and between years. Therefore, *C. crangon* was used as a model organism to study physiological and evolutionary adaptations that allow organisms to cope with variable environmental conditions. This knowledge, in turn, can help to assess, how organisms are affected by future trends, such as predicted by climate change scenarios. This thesis focusses on the energy (lipid) storage capacity and the metabolic flexibility in view of protein and enzyme polymorphism of *C. crangon*.

Most decapod crustaceans accumulate lipid depots in the midgut gland, which are used for reproduction and to overcome periods of food scarcity. Such lipid reserves mainly consist of neutral lipids, e.g. triacylglycerols. In times of energy demand, those triacylglycerols are hydrolyzed to glycerol and fatty acids. The fatty acids are transferred by the action of enzymes and transport proteins from lipid depots to different organelles to be catabolized. However, brown shrimps seem to follow a different energy utilization strategy. They store only low quantities of lipids in their midgut glands (14 to 17% of dry mass, DM) compared to other decapod shrimps that inhabit the North Sea, e.g. the pink shrimp *Pandalus montagui* which stores up to 47 to 70%DM in the midgut gland. Most lipids in the midgut gland of *C. crangon* are phospholipids, which usually serve as structural components in biomembranes rather than storage compounds. Hence, brown shrimps use the midgut gland as a metabolic center rather than a depot organ. The limited lipid storage capabilities may be related to mutations in the binding site of the fatty acid transport proteins and to alterations of key anabolic pathways, which are involved in the formation of triacylglycerols.

Possible disadvantages may be compensated by an extraordinary genetic potential. *C. crangon* has one of the largest genomes of the decapods and with 11.4 pg DNA in a single haploid cell (C value) it is even more than three times larger than the human genome. Our transcriptome analyses revealed a high and variable level of polymorphism of digestive enzymes. Lipolytic, proteolytic, and glycolytic enzymes were expressed in up to 13 isoforms.

The degree of polymorphism varied among enzymes: higher numbers of isoforms appeared in enzymes with diverse extra- and intracellular functions. The limited lipid storage capacity may be compensated by the function of the midgut gland as a metabolic center, which instantly provides the energy necessary for various biological processes. Expression of triacylglyerol lipases and phopholipases followed a seasonal cycle, reflecting the utilization of alimentary lipids in summer and autumn. Elevated expression of calcium-independent phospholipase A_2 (which may play a major role in membrane phospholipid remodeling) and the seasonal course of the gene expression ratios between triacylglycerol (TAG)-lipases and phospholipases indicate utilization of intracellular phospholipids in winter and spring, when food is scarce.

Mutated and thus at least partially malfunctional serine proteinases are compensated by the expression of up to twelve isoforms of the cysteine proteinase cathepsin L. The pattern of proteinase polymorphism is not gender or food-related. It is even variable within individuals from the same population. Metabolite profiles are, however, not affected, independent of prevailing serine or cysteine proteinases. This suggests that *C. crangon* does not face metabolic stress due to the lack of serine proteinases and it is capable of fulfilling its metabolic requirements with the support of cysteine proteinases.

The high degree of enzyme polymorphism apparently enables *C. crangon* to utilize a wide spectrum of substrates as energy sources, and to respond flexible to metabolic challenges in terms of energy utilization and allocation. Molecular alterations and enzyme polymorphism may result from a high mutation rate, which is characteristic of organisms with a huge genome, or from alternative splicing processes. Such a high polymorphism level of digestive and metabolic enzymes in *C. crangon* can be regarded as an important trait that enhances the fitness of an organism in a variable environment.

ZUSAMMENFASSUNG

Die Nordseegarnele, *Crangon crangon*, kommt an fast allen europäischen Küsten vor. Sie weist jedoch in den flachen Küstengebieten der südlichen Nordsee die höchsten Dichten auf. Hohe Abundanzen und hohe Reproduktionsraten machen diese Art zu einem wichtigen Bestandteil küstennaher Ökosysteme und zu einer wertvollen Zielart für die lokale Fischerei. Der biologische Erfolg von *C. crangon* in der Nordsee lässt darauf schließen, dass diese Art in Bezug auf abiotische und biotische Faktoren, bei denen sich auch Menge und Qualität der Nahrung mit den Jahreszeiten und zwischen den Jahren schnell ändern können, gut an variable Umweltbedingungen angepasst ist. Daher wurde *C. crangon* als eine geeignete Art ausgewählt, um physiologische und evolutionäre Anpassungen zu untersuchen, die es Organismen ermöglichen, mit variablen Umweltbedingungen umzugehen. Dieses Wissen kann wiederum dazu beitragen, die Wirkung zukünftiger Klimaentwicklungen auf Organismen abzuschätzen. Die vorliegende Arbeit konzentriert sich daher auf die Energiespeicherkapazität (in Form von Lipiden) und metabolische Flexibilität der Nordseegarnele im Hinblick auf Protein- und Enzympolymorphismus.

Die meisten Zehnfußkrebse (Decapoda) reichern in der Mitteldarmdrüse Lipiddepots an, die für Fortpflanzungsprozesse und zur Überwindung von Hungerphasen genutzt werden. Solche Lipidreserven bestehen hauptsächlich aus Neutrallipiden, z.B. Triacylglycerol (TAG). In Zeiten des Energiebedarfs werden diese TAGs zu Glycerin und Fettsäuren hydrolysiert. Die Fettsäuren werden durch Enzyme auf Transportproteine übertragen und von den Lipiddepots zu verschiedenen Organellen verbracht, wo sie abgebaut werden. Die Nordseegarnele scheint dabei jedoch eine Sonderrolle einzunehmen und einer anderen Strategie zur Energienutzung zu folgen. Im Vergleich zu anderen Krebsarten in der Nordsee (z.B. *Pandalus montagui* mit 47-70% Lipid an der Trockenmasse, TM) verfügt sie nur über geringe Mengen an Lipiden in der Mitteldarmdrüse (14-17%TM). Die meisten dieser Lipide sind Phospholipide, die gewöhnlich als Strukturlipide in Biomembranen und nicht als Speicherlipide fungieren. Die Mitteldarmdrüse der Nordseegarnele hat also eher die Funktion eines zentralen Stoffwechselorgans als die eines Speicherorgans. Die begrenzten Lipidspeicherkapazitäten bei *C. crangon* können mit Mutationen an der Bindungsstelle der Fettsäuretransportproteine und mit Veränderungen der wichtigsten anabolischen Stoffwechselwege zusammenhängen, die beim Aufbau von TAG eine Rolle spielen.

Mögliche Nachteile scheinen durch ein außergewöhnliches genetisches Potenzial ausgeglichen zu werden. *C. crangon* hat eines der größten Genome innerhalb der Dekapoden. Es umfasst 11,4 pg DNA in einer einzelnen haploiden Zelle (C-Wert) und ist damit mehr als dreimal so groß wie das menschliche Genom. Die Transkriptomanalyse der Mitteldarmdrüse ergab dabei einen hohen und variablen Grad an Polymorphismus bei den Verdauungsenzymen. Lipolytische, proteolytische und glykolytische Enzyme wurden in bis zu dreizehn Isoformen exprimiert. Der Grad des Polymorphismus variierte zwischen den Enzymen: In Enzymen mit unterschiedlichen extra- und intrazellulären Funktionen trat dabei eine größere Anzahl von Isoformen auf. Auf diese Weise könnte die begrenzte Lipidspeicherkapazität durch hohe Stoffwechselraten der Mitteldarmdrüse ausgeglichen werden, die sofort die für die verschiedenen biologischen Prozesse notwendige Energie liefert.

Die Expression von TAG-Lipasen und Phospholipasen folgt einem saisonalen Zyklus, der die Verwendung von Nahrungslipiden im Sommer und Herbst widerspiegelt. Die erhöhte Expression von calciumunabhängiger Phospholipase A_2 (die eine wichtige Rolle beim Umbau von Membranphospholipiden spielen kann) und der saisonale Verlauf der Genexpressions-verhältnisse zwischen TAG-Lipasen und Phospholipasen deuten auf die Nutzung intrazellulärer Phospholipide im Winter und im Frühling hin, wenn die Nahrung knapp ist.

Mutierte und damit zumindest teilweise eingeschränkte Serinproteinasen werden darüber hinaus durch die Expression von bis zu zwölf Isoformen der Cysteinproteinase Cathepsin L kompensiert. Das Muster des Proteinase-Polymorphismus ist dabei nicht geschlechts- oder nahrungsabhängig. Es variiert sogar zwischen Individuen aus derselben Population. Die Profile der Metaboliten sind jedoch nicht betroffen, unabhängig von den vorherrschenden Serin- oder Cysteinproteinasen. Dies deutet darauf hin, dass *C. crangon* keinem metabolischen Stress aufgrund des Mangels an Serinproteinasen ausgesetzt ist und die Stoffwechselanforderungen mithilfe von Cysteinproteinasen erfüllen kann.

Der hohe Grad an Enzympolymorphismus ermöglicht es *C. crangon* anscheinend, ein breites Spektrum von Substraten als Energiequelle zu nutzen und flexibel auf metabolische Herausforderungen hinsichtlich der Energienutzung und -allokation zu reagieren. Molekulare Veränderungen und Enzympolymorphismus können auf eine hohe Mutationsrate, die für Organismen mit großem Genom charakteristisch ist, oder auf Prozesse des „alternative splicing“ zurückzuführen sein. Zusammenfassend kann der Polymorphismus von Verdauungs-

und Stoffwechselenzymen bei *C. crangon* als ein Schlüsselmerkmal angesehen werden, das die Fitness eines Organismus in einer variablen Umgebung erhöht.

CHAPTER 1

SCIENTIFIC BACKGROUND AND OBJECTIVES

Nature impresses us by the richness of lifeforms that emerged in the most diverse habitats. This variety, in turn, gives us an idea about the incredible power and consistency of the drivers that promote biological diversification. The evolution of fundamental structures and biochemical interactions to maintain life under diverse conditions is a perpetual and comprehensive process acting at all levels of living systems (Hochachka & Somero 2002). Challenges imposed by environmental conditions to biochemical systems are very diverse, especially in habitats, where conditions change frequently and rapidly. In this sense, organisms with a high phenotypic plasticity may have advantages to survive and to prosper. Here I refer to phenotypic plasticity as the ability of one genotype to produce more than one phenotype in terms of gene expression and biochemical functions, rather than morphological and anatomical varieties (Kelly et al. 2012).

It is generally accepted that heterogeneous and variable environments demand a high degree of physiological flexibility from the organisms living there. In turn, organisms living in a variable habitat are expected to show high abilities for adaptation (e.g. Wilcox & Jeffries 1974; Nelson & Hedgecock 1980; Abdullah & Shukor 1993; Saborowski et al. 2012). Identifying mechanisms of molecular adaptation can provide insights into the process of phenotypic evolution. However, it may be difficult to quantify the phenotypic effects of specific mutational changes (Storz & Zera 2011). Eventually, it is necessary to verify the adaptive significance of genetically based changes in protein function. In terms of enzymes, this can comprise variation in activity and metabolic performance. This is, however, difficult to achieve under in vivo conditions. A more general approach is to investigate the functionality of enzymes and other proteins by means of sequence and structure analysis. In this respect, transcriptome-based analyses are powerful tools for elucidating the molecular mechanisms that underlie the ability of organisms to sustain and to adapt to the conditions in dynamic and changing environments (Lockwood et al. 2015).

Moreover, the ongoing global warming process will significantly and rapidly alter habitat characteristics. Amongst others, coastal and shelf ecosystems are most vulnerable. They will be affected by temperature increase, hydrodynamic processes, and, finally, regime shifts and significant changes of species composition. The North Sea experienced a continuous temperature increase of 1.7°C in the last decades (Wiltshire & Manly 2004), which goes along

with a distinct regime shift and other significant biological and hydrological changes (Beaugrand 2004; Opdal et al. 2019).

The brown shrimp *Crangon crangon* (Linnaeus, 1758) is an interesting model in terms of phenotypic plasticity. It has high reproduction rates and appears well adapted to a dynamic environment. Moreover, it shows extraordinary molecular and biochemical characteristics, which are apparently not common among crustacean species (Teschke & Saborowski 2005; Saborowski et al. 2012). These properties encouraged the start of this investigation with the aim of studying adaptive properties of organisms, which appear beneficial in a variable and changing environment like the North Sea.

The following paragraphs will first present the biological characteristics of *C. crangon* in terms of morphology, ecology, and physiology. The final paragraph of this introduction will summarize the objectives and specify the aims and goals of this study.

1.1 The brown shrimp in a heterogeneous and variable environment

1.1.1 Systematics and morphology

The brown shrimp, *Crangon crangon* (Linnaeus, 1758), is a decapod crustacean. Within the suborder Pleocyemata, it belongs to the infraorder Caridea and the family Crangonidae. The genus *Crangon* comprises 19 recent species, inhabiting exclusively the northern hemisphere. *C. crangon* has an elongated shrimp-like body shape, with a clearly defined cephalothorax and pleon (Fig. 1). The animals grow discontinuously and gain mass and length after frequent molts. They may reach a total length of almost 80 mm weighing about 3.5 g (wet mass), but the market size barely exceeds 65 mm and 2 g. In nature, shrimp may reach a maximum age of three years (Oh et al. 1999). However, the majority of animals is harvested at the end of the first year or the beginning of the second year.

Fig. 1. The brown shrimp *Crangon crangon.*

1.1.2 Distribution

Brown shrimps inhabit areas within a wide latitudinal range in Europe (Fig. 2). They are found in fully marine and oligohaline waters as well as brackish lagoons and estuaries. They are present in the White Sea and along the Scandinavian, western European and Iberian coasts. They are less frequent in the Mediterranean and Black Sea (Holthuis 1980; del Norte-Campos & Temming 1994; Campos 2009). Since 2003 *C. crangon* appeared in Iceland and rapidly colonized its southern and western coasts (Gunnarsson et al. 2007). The highest densities of shrimps are present in the southern North Sea and around the British Isles. Variable and occasionally extremely high abundances of max. 82 specimens per m^2, including juveniles, were reported (Boddeke et al. 1986). The long-term mean density of *C. crangon* along the German coast in autumn is 1.4 individuals per m^2. Single sampling events exceeded densities of 12 animals per m^2 (Siegel et al. 2005).

C. crangon can be found mainly on shallow marine and estuarine soft bottom areas down to about 40 m (Pinn & Ansell 1993; Siegel et al. 2005). However, it also occurs at depths of up to 90 m (Al-Adhub & Naylor 1975; Hinz et al. 2004). In the North Sea, adult brown shrimp show seasonal migration patterns. During summer, the majority of the population inhabits the shallow and nutrient-rich areas of the Wadden Sea. In winter, they migrate to deeper waters (Boddeke 1976). Drivers of this migration might be changes in temperature, salinity as well as predator pressure and food availability (Siegel et al. 2005).

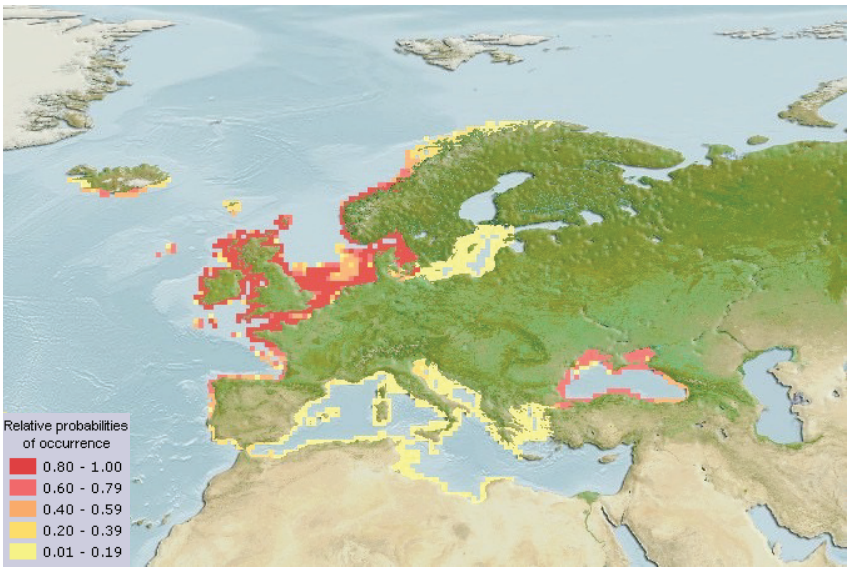

Fig. 2. Computer-generated distribution map for *Crangon crangon*.
(www.aquamaps.org, version of Aug. 2016. Web. Accessed 28 Aug. 2019)

1.1.3 Population genetics

Four major phylogeographic groups have been identified for the brown shrimp *C. crangon*, the northeastern Atlantic, the western Mediterranean, the Adriatic and the Black Sea group. It seems that gene flow between these four groups is virtually absent and it is primarily established by oceanographic barriers (Luttikhuizen et al. 2008). Of the four major groups, the western Mediterranean population is the oldest and demographically most stable one, having a history of up to one million years (Luttikhuizen et al. 2008). Therefore, it is most likely that the northeast Atlantic population originates from the western Mediterranean population.

This study focuses on the populations in the southern North Sea that, according to the genetic studies of Luttikhuizen et al. (2008), belongs to the same phylogeographic group as all other populations of *C. crangon* in the northeast Atlantic.

1.1.4 Reproduction

C. crangon specimens mature at a size of 30-40 mm (total length), depending on external temperature conditions (Tiews 1970). After internal insemination, females store sperm and carry their fertilized eggs beneath the abdomen until they hatch (Boddeke et al. 1991). *C. crangon* produce eggs throughout the year, with high abundances of egg-bearing females present from winter to late summer but only few in autumn (Boddeke 1982; Temming & Damm 2002; Siegel et al. 2005; Hünerlage et al. 2019). Individual females may produce two or more clutches per season (Lloyd & Yonge 1947; Clarke 1979b).

Brood size is not as closely related to the size of the female as in other shrimp species, e.g. *Pandalus montagui* (Leach 1814). Brood size of *C. crangon* ranges between 1000 and 14,000 eggs per female. *C. crangon* is more a r- strategist than a K-strategist, meaning that they produce a large number of small eggs, rather than fewer but larger eggs. This strategy can be seen as more efficient, when food availability for planktonic offspring is unpredictable (Vance 1973; Clarke 1979b). Egg size depends on the size of the female, with larger females normally producing larger eggs (Campos 2009). Additionally, egg size depends on the season. Eggs produced in winter are usually larger than those produced during summer (Havinga 1930). The fresh mass per egg varies between 70 and 80 µg.

Embryogenesis depends on the temperature. At 6°C it takes between ten and twelve weeks until hatching, but about three weeks at 18°C (Tiews 1970). After hatching, the free-floating planktonic larvae pass through six different stages (Ehrenbaum 1890; Williamson 1960; Dalley 1980). Development of the pelagic larvae to juveniles last about three to seven weeks, again depending on temperature (Criales & Anger 1986). Shrimp larvae migrate after the first planktonic stages to shallow nursery areas, such as estuaries, to grow up (Boddeke 1976; Beukema 1992; Campos 2009). The post-larvae settle on muddy and sandy grounds and continue with their juvenile and adult stages as members of the epibenthic community (Campos et al. 2012).

Juveniles originating from winter eggs move into shallow waters in late spring or early summer (Temming & Damm 2002), where they may have access to abundant food and avoid predators (Boddeke 1989). When they grow up, the shrimps move again to deeper waters. The migration of *C. crangon* is related to the size of the individual. They start migrating to deeper waters when their body length reaches about 20 mm. In addition, migration activity is also influenced by sex, since females grow faster than males (Boddeke 1976; Beukema 1992; Hufnagl et al. 2010).

C. crangon is capable of changing sex, however, the low number of sex reversals indicates that this species is a facultative hermaphrodite (Martens & Redant 1986; Boddeke et al. 1991; Schatte & Saborowski 2006) and egg production of sex changers is negligible (Hufnagl et al. 2010).

1.1.5 Feeding and nutrition

The brown shrimp has been described as a trophic generalist without specific feeding preferences. Their feeding habits are omnivorous and carnivorous, opportunistic with often cannibalistic behavior (Kuhl 1972; Marchand 1981; Pihl & Rosenberg 1984). The food spectrum of *C. crangon* in the Elbe estuary comprised a wide range of smaller benthic crustaceans, molluscs, polychaetes, and fish remains, but also green algae, mud, and detritus (Plagmann 1939).

Digestion in crustaceans is a complex process, based on various morphological, anatomical and physiological peculiarities. For hydrolyzing food items, crustaceans express a set of digestive enzymes, which include proteinases, lipases, esterases, and glucanases (Dall & Moriarty 1983; Saborowski et al. 2012). The dominating proteinases in the digestive tracts of most crustaceans are serine proteinases such as trypsin and chymotrypsin (Ceccaldi 1998; Saborowski et al. 2004). Most of *C. crangon* individuals, however, express predominantly cysteine proteinases and only about 10% of the individuals show significant levels of serine proteinases (Saborowski et al. 2012). The main difference between serine and cysteine proteinases is the catalytic site and, thus, cleavage preferences. As the name suggest, serine proteinases have a serine residue in its active site, cysteine proteinases have a cysteine, and therefore their mechanism of action is different. In addition to the unusually low activity of serine proteinases, *C. crangon* also show a high level of polymorphism in digestive enzymes, such as proteinases (Saborowski et al. 2012) and lipase/esterase (Saborowski, pers. comm.).

1.1.6 Ecological impact

The role and function of *C. crangon* in the ecosystem is given by its distribution range (Campos 2009). In the North Sea, the brown shrimp plays a key role in the ecosystem due its high abundance. Therefore, the high density of the brown shrimp has a strong impact on benthic shallow-water communities (Campos et al. 2012). Predators of *C. crangon* benefit from its high abundance; some of these predators include fish, crustaceans and wading birds (Del Norte-Campos & Temming 1994; Walter & Becker 1997).

C. crangon is a dominant component of the epibenthic community in the southern North Sea. Its high abundance and high reproduction rates suggest that this species is well adapted to the local environmenttal conditions. The brown shrimp exhibits high tolerance and adaptability since it is regularly confronted with a high degree of seasonal temperature variability. During the winter season, temperatures can fall below 0°C, and, the brown shrimp

seems to equally tolerate low temperatures independently of its life stages (Reiser et al. 2014). Fluctuations in abundance follow a seasonal pattern, which is related to their migration patterns and life cycle. Abundance of juveniles is higher in shallow water during spring and summer in comparison with autumn and winter (Campos et al. 2012).

1.1.7 Fisheries

C. crangon fisheries in coastal areas of the North Sea has a long tradition of hundreds of years (Detlefsen 1984). First evidence of small-scale fisheries was documented in the 17th century (Lotze 2007). Until the early 19th century, exploitation did not exceed subsidiary relevance, but advanced rapidly when sailing boats and, later, engine-driven boats were deployed during sea-going trawling (Hünerlage et al. 2019).

At present, *C. crangon* represent the most important target of coastal fisheries in the southern North Sea. The Netherlands, Germany, and Denmark contribute most to its economical exploitation (ICES 2015). Average landings increased continuously since 2000, being 40% higher nowadays than in previous decades. Only in 2014, *Crangon* fisheries yielded a record of 128 million euros (Scientific, Technical, and Economic Committee for Fisheries 2016). Concomitant with the increase in landings, its natural mortality by predations has decreased (Tulp et al. 2016).

Surprisingly, *C. crangon* populations in the North Sea appear remarkably stable against high fishing pressure (Neudecker et al. 2007). Siegel et al. (2005) analyzed fisheries data for the German Bight from 1972 to 2002. Although the authors found strong year-to-year variations in *C. crangon* densities, the results showed neither a declining nor an increasing trend. Year-to-year variations in the *C. crangon* stock are common and have also been reported from the Dutch Wadden Sea (Cattrijsse et al. 1997).

1.2 The reference species *Crangon allmanni* and *Pandalus montagui*

The southern North Sea is a highly dynamic area with high productivity and significant fresh water discharges from the rivers Eider, Elbe, Weser, Ems and Oosterschelde. Many other species of crustaceans inhabit this ecosystem. In order to better identify and understand the biochemical characteristics, which support adaptive physiological processes in *C. crangon*, I include *Crangon allmanni* (Kinahan, 1860), which is closely related to *C. crangon*. *C. allmanni* is smaller, less abundant and often confused with *C. crangon* due to their similarity. The

distinctive mark of *C. allmanni* is a groove on the dorsal side of the 6th abdominal segment (Fig. 3). Information about its ecology and life cycle is surprisingly scarce.

The only population of *C. allmanni* in the inner German Bight is located south of the island of Helgoland (Blahudka & Türkay 2002). The life span of *C. allmanni* lasts for about 1.5 years maximum. Recruitment takes place mainly from September to November (Blahudka & Türkay 2002). In contrast to *C. crangon*, little information is available about the biology of *C. allmanni*. A synopsis of the biology of *C. allmanni* from Northumberland waters has been given by Allen (1960). Migration of *C. allmanni* is still under debate. It has been suggested that they migrate to shallow coastal waters in October and back offshore in late spring (Allen 1960). However, according to more recent studies, the rapid decline in abundance towards summer is due to high mortality (Creutzberg & van Leeuwen 1980). In July and August, the population is composed mainly of females without eggs. A maximum number of ovigerous females occurs from March to June (Blahudka & Türkay 2002).

C. allmanni is very sensitive to environmental stress, and prefers colder conditions than *C. crangon* (Allen 1960; Teschke & Saborowski 2005). According to Blahudka & Türkay (2002), the principal environmental factor that *C. allmanni* requires to form stable populations is a constantly high salinity at oceanic level. *C. allmanni* is considered to live in a quite stable environment since it is found in more than 20 m depth, while *C. crangon* is considered to live in a variable environment since it is found in the fluctuating environment of the intertidal area and estuaries (Abdullah & Shukor 1993).

Pandalus montagui (Leach, 1814) commonly known as pink shrimp, is spread over the northern hemisphere from Greenland, Iceland, North Sea to Norway (Fig. 3). Pink shrimps, like brown shrimps, perform a seasonal migration. However, it differs from *C. crangon* in several aspects, e.g. in reproductive effort, thermal tolerance window (Simpson et al. 1967) and sex change. *P. montagui* is a protandric hermaphrodite, which means that the individual with male characteristics first reproduces as males, but after the first year, they change their sex, when male sexual characteristics transform into female characteristics. Thereafter, they are able to reproduce as females in their second year of life (Sabel et al. 2017). This species can reach up to 80 mm length. However, the males are usually smaller than the females (Mistakidis 1957). Since secondary females live as males the first year of their life, they are smaller than primary females.

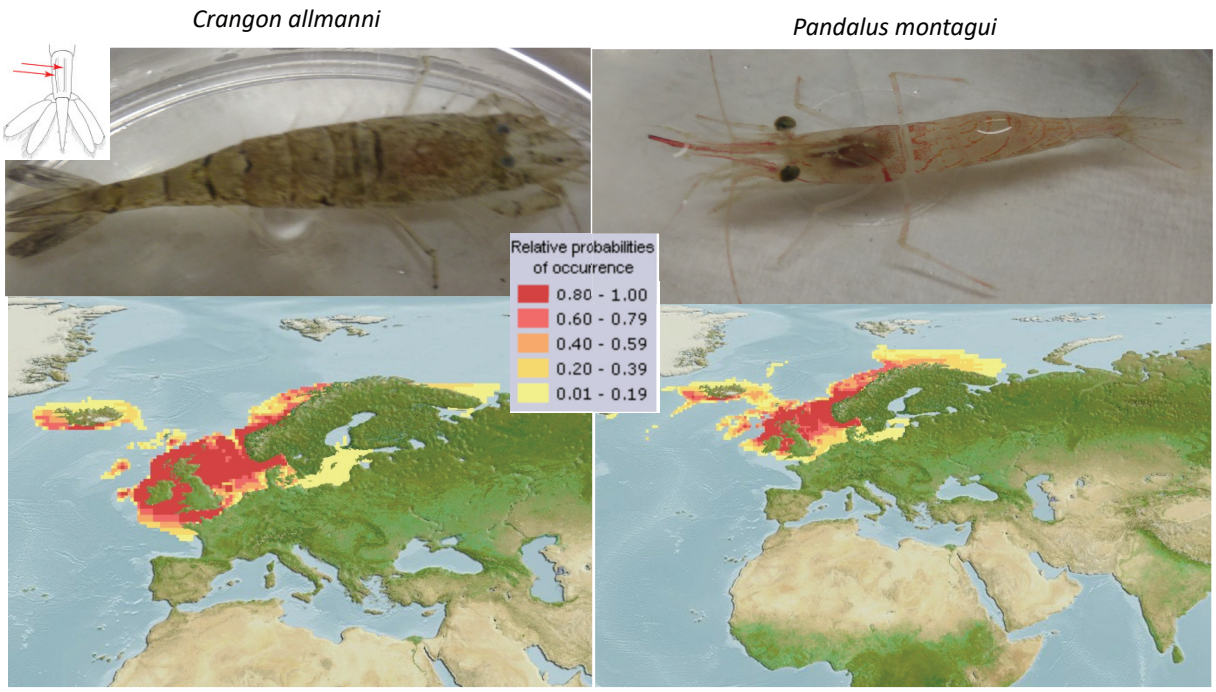

Fig. 3. *Crangon allmanni* (top left) and its distribution (bottom left). *Pandalus montagui* (top right) and its distribution (bottom right) (www.aquamaps.org, Web. Accessed 06 Sep. 2019).

1.3 Enzyme/protein polymorphism and biochemical adaptation

Adaptations of organisms to specific environmental conditions start at the molecular level. This can include diverse molecular steps from control of gene expression or metabolic regulation to the evolutionary origin of new classes of proteins. Recent studies of regulation of gene expression, metabolic biochemistry, protein structure-function relationships, genomics and proteomics have been essential to gain deeper knowledge about important issues concerning evolution, diversity, ecology, and biogeography (Hochachka & Somero 2002). Here I refer to adaptation as a trait that enhances the fitness of an organism, and the current beneficial characteristic reflects the selective advantage of the trait at its time of origin. In this sense the high polymorphism level characteristic of *C. crangon* can be considered as a biochemical adaptation (Kimura & Ohta 1971; Nelson & Hedgecock 1980; Tomaiuolo et al. 2008).

Polymorphism is a term normally used, when members of a population present distinct phenotypes in terms of a particular characteristic of an enzyme or protein (Harris 1971). Protein polymorphism is a phase of molecular evolution. It represents the first step in the long-lasting establishment of mutations within populations (Kimura & Ohta 1971). In this way, adaptive polymorphism may represent a suitable parameter for studying evolutionary processes in variable habitats (Nelson & Hedgecock 1980). In brown shrimps, unspecific feeding preferences

may be supported by an elevated polymorphism of digestive enzymes. Saborowski et al. (2012) suggested that the polymorphism of proteinases from the midgut gland of the brown shrimp seems to reflect a highly adaptive potential to variable trophic conditions. Therefore, high polymorphism in digestive enzymes of *C. crangon* may be a good example of a molecular adaptation of an organism to a heterogeneous and changing environment (Van Wormhoudt et al. 1995; Le Moullac et al. 1996; Sellos et al. 1998).

1.4. Justification

Environmental change is a continuous and dynamic process. Everything that exists nowadays is the result of millions of years of evolution at different levels. Understanding how different species cope with their environment can help us to learn more about evolutionary processes, i.e. how they became what they are. Even more important nowadays is the prediction of their capacities to respond and adapt to stress depending on their evolutionary history.

In this sense, the brown shrimp represents an important subject of study. It is well adapted to changing environmental conditions, as the high reproduction rates and the unspecific feeding preferences have shown. Additionally, it represents an important economical source for German fisheries. Most important for the purpose of this work, it shows highly interesting molecular properties, hence the species deviates from most other crustaceans, as shown by their high level of polymorphism and their substitution of some of the most important digestive enzymes. Therefore, the justification of this work relies on the economical, ecological and evolutionary importance of knowing the molecular particularities that support the adaptation of the brown shrimp to a highly variable environment.

Biology and ecology of *C. crangon* were intensively studied in the past. However, there is still a significant lack of understanding, which physiological and biochemical processes support the high plasticity and, thus, the exceptional ecological success of *C. crangon*. In this study, I will focus on genes and gene products, respectively, with high functional relevance in energy utilization and storage.

1.5 Objectives and approaches

The overall goal of the present work is to study and to identify biochemical properties of *C. crangon*, which are capable of supporting adaptive physiological processes and strategies in a highly variable and changing environment. The following five objectives were defined.

1.5.1 Objective 1

To investigate the biochemical and physiological aspects of lipid storage capacities and nutritional preferences of North Sea shrimps by determining:

- seasonal cycles of lipid storage in the midgut glands of *C. crangon* and *P. montagui*
- lipid class and fatty acid compositions of both species
- feeding preferences by applying the fatty acid trophic marker (FATM) concept.

1.5.2 Objective 2

To assess the variability and polymorphism of selected digestive enzymes in *C. crangon* through putative isoforms by:

- obtaining the whole transcriptome of the midgut gland of *C. crangon*
- determining putative isoforms of enzymes involved in lipid, protein and carbohydrate metabolism.

1.5.3 Objective 3

To assess the biochemical aspects of lipid metabolism of the midgut gland of *C. crangon* by:

- defining the seasonal expression patterns of key lipase digestive enzymes
- analyzing the sequences of fatty acid binding proteins (FABPs) from the transcriptome of *C. crangon*
- predicting the 3D structure of the FABPs found in the transcriptome of *C. crangon*.

1.5.4 Objective 4

To investigate the substitution of serine proteinases by cysteine proteinases at the molecular level by analyzing:

- trypsin sequences from the transcriptome of *C. crangon*
- trypsin sequences from gene expression analysis
- primary and secondary structure of trypsin isoforms.

1.5.5 Objective 5

To identify the metabolic profiles of *C. crangon* midgut glands and to compare the metabolite compositions between individuals with high and low trypsin activity by:

- selecting *C. crangon* individuals with high and low levels of trypsin activity in the midgut gland
- obtaining the metabolite profiles of the midgut gland of *C. crangon*
- comparing metabolite compositions between individuals with high and low trypsin activity.

CHAPTER 2

MATERIAL & METHODS

This section contains information about materials and methods used in this thesis which are not explicitly outlined in the two publications and the manuscript. It includes an overview of the study area in the southern North Sea and the sampling sites. It also contains details of tissue collection, enzyme assays, and the extraction of metabolites for ^{1}H-NMR spectroscopy, which is addressed in chapter 4.

2.1 Animal collection

Crangon crangon specimens were caught by bottom trawling with RV *Uthörn* in the German Bight near the island of Helgoland and in the outer Weser estuary in 2015, 2016 and 2017 (Table 1).

Table 1. Sampling dates of *C. crangon* analyzed for this study.

Year	Month	Day	Thesis chapter	Publication
2015	June	26	3	2
2016	February	19	3	1 & 3
2016	April	18	3	1 & 3
2016	July	19,20,21	3	1 & 3
2016	October	24,25,26	3	1 & 3
2017	May	23	4	Unpubl.
2017	June	6,23	4	Unpubl.
2017	July	7	4	Unpubl.

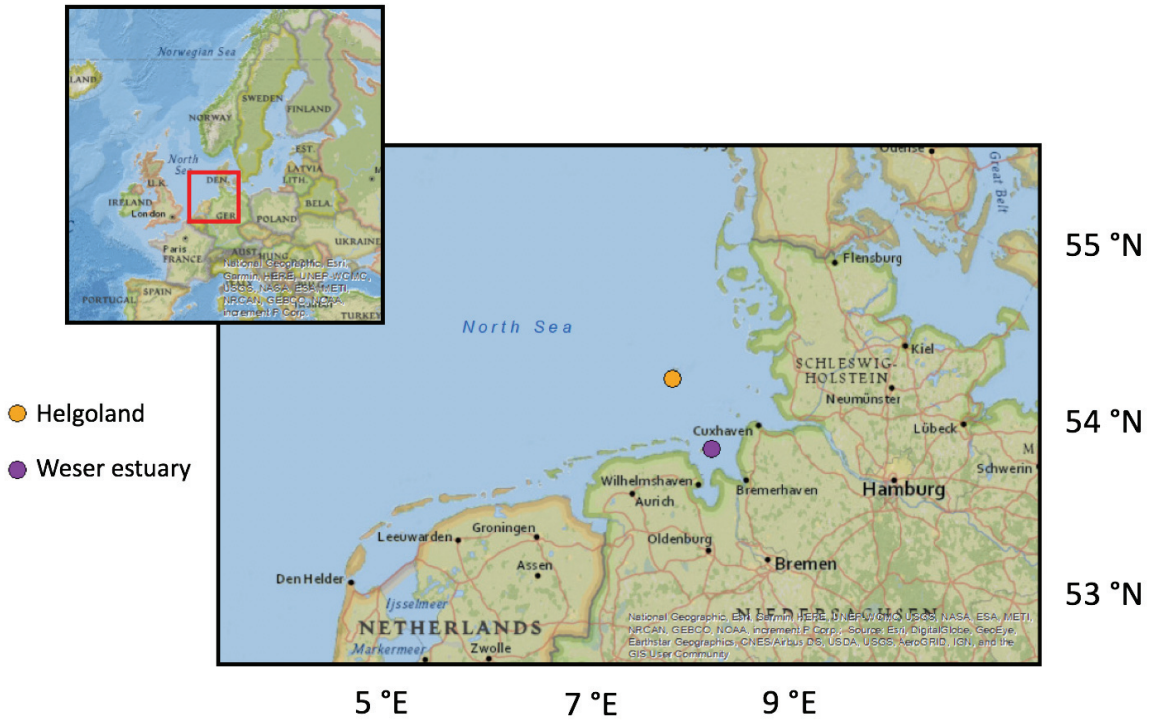

Fig. 4. North Sea sampling locations of individuals analyzed for publications 1 & 3.

2.2 Tissue collection and trypsin activity

After collection, total body length (rostrum to tip of telson) and sex of the animals were determined. The midgut glands of 100 *C. crangon* specimens were dissected. Each midgut gland was divided into two subsamples, each transferred to a 1.5-ml reaction tube, shock-frozen in liquid nitrogen, and stored at -80°C until extraction.

One of the two subsamples was used to analyze trypsin activity. The sample was homogenized in 500 µl of milliQ water on ice. The homogenate was centrifuged for 12 min at 4°C and 15,000 *g* to separate lipids and other tissue debris. The aqueous supernatant containing the enzymes was stored at -80°C. The supernatants were analyzed for protein concentration after Bradford (1976), using bovine serum albumin as a protein standard. Samples were placed on ice during the entire procedure. Trypsin activity was quantified using a synthetic substrate Na-benzoyl-DL-arginine-p-nitroanilide (BAPNA: B4875; Sigma-Aldrich). 50 µl of enzyme extract was placed in a microplate well with 250 µl of 1.2 mM substrate in a 0.1 M Tris-HCl, pH 8.0, containing 10 mM calcium chloride. The absorbance at 405 nm was recorded every 5 min for 30 min at room temperature in a microplate reader. Specific activity was calculated as arbitrary activity units (AU = difference of absorbance per min and per mg of protein). All assays were done in triplicate. From the 100 individuals analyzed, 15 individuals

were chosen, five with low, five with medium and five with high trypsin activity. The remaining subsamples of these 15 individuals were used for the extraction of polar metabolites.

2.3 Extraction of polar metabolites

Polar metabolites were extracted from the midgut gland of *C. crangon* following a two-step extraction protocol after Wu et al. (2008). Pre-weighed frozen samples (50-100 mg) of midgut gland tissue were added to homogenization tubes containing 400 µl ice-cold methanol and 125 µl ice-cold Milli-Q water. The samples were kept on ice during the entire procedure. Cell lysis was done by ultrasonication (Branson Sonifier Cell Disruptor) with three bursts of 5 s and 10 s breaks in between. Subsequently, 400 µl of chloroform and 400 µl of Milli-Q water were added to each sample and mixed for 15 seconds. After 10 min on ice, the samples were centrifuged at 3000 *g* at 4°C for 10 min. The upper layer, which contained the polar part, was transferred to a new 2-ml reaction tube and dried overnight at room temperature in a rotational vacuum dryer (Eppendorf Concentrator 5301).

The dried samples were re-suspended in D_2O containing 0.05% trimethylsilyl propionate (TSP; Sigma-Aldrich, St. Louis, USA). The samples were transferred into a standard 50 µl zirconia on rotor of a triple tuneable ^{1}H-^{31}P-^{31}C HRMAS probe. Sample spinning rate was 3000 Hz at 20°C. Data were recorded with TOPSIN 3.2 software (Bruker-BioSpin GmbH, Germany).

2.4 ^{1}H-NMR spectroscopy

The NMR protocol includes different techniques (Schmidt et al. 2017). The ^{1}H-HRMAS NMR spectra for the midgut gland samples were obtained with a 9.4 T NMR spectrometer (Avance III HD 400 WB, Bruker-Biospin GmbH, Germany). The Call-Purcell-Meiboom-Gill (CPMG) sequence was used for metabolite analysis and quantification. Metabolite identification and quantification were done with Chenomx NMR suite 8.1 software (Chenomx Inc., Edmonton, Canada). In all spectra, peaks were assigned to metabolites by their chemical shifts using the Chenomx database

CHAPTER 3

PUBLICATIONS

3.1 Outline and contributions

This session, explain the participation of all co-authors of the three manuscripts that are part of this thesis and describes the contribution of the first author in % of the total load.

Publication I

Diana Martínez-Alarcón, Reinhard Saborowski, Eleni Melis, Wilhelm Hagen (2019)

Seasonal lipid dynamics of the shrimps *Crangon crangon* and *Pandalus montagui* in the German Bight (North Sea). *Marine Ecology Progress Series* 625:41-52

The idea, design and practical work of this research was performed by myself with the scientific advice of WH and RS. EM assisted in the analysis of samples. The manuscript was written by myself with the scientific support of WH and RS.

Contribution of the candidate in % of the total work load (up to 100% for each of the following categories):

Experimental concept and design:	ca. 80%
Experimental work and/or acquisition of (experimental) data:	ca. 90%
Data analysis and interpretation:	ca. 85%
Preparation of figures and tables:	ca. 95%
Drafting of the manuscript:	ca. 80%

Publication II

Diana Martínez-Alarcón, Lars Harms, Wilhelm Hagen, Reinhard Saborowski (2019)

Transcriptome analysis of the midgut gland of the brown shrimp *Crangon crangon* indicates high polymorphism in digestive enzymes. *Marine Genomics* 43:1-8.

This research was designed and performed by myself under the scientific advice of RS and WH. Bioinformatic analyses were made by LH and myself. The biological data was interpreted by RS and myself. I wrote the draft of the manuscript and revisions were made by all co-authors.

Contribution of the candidate in % of the total work load (up to 100% for each of the following categories):

Experimental concept and design:	ca. 80%
Experimental work and/or acquisition of (experimental) data:	ca. 80%
Data analysis and interpretation:	ca. 80%
Preparation of figures and tables:	ca. 90%
Drafting of the manuscript:	ca. 85%

Publication III

Diana Martínez-Alarcón, Wilhelm Hagen, Christoph Held, Reinhard Saborowski

Molecular aspects of lipid metabolism in the midgut gland of the brown shrimp *Crangon crangon.* *Comparative Biochemistry and Physiology Part B: Biochemistry & Molecular Biology: to be submitted*

The concept of the study was elaborated by RS, WH and myself. I performed the molecular analyses under the advice of CH and RS. Data analysis and interpretation of the results were done by RS and myself. I drafted the manuscript and RS assisted during the entire process. All co-authors participated in the revisions of the manuscript.

Contribution of the candidate in % of the total work load (up to 100% for each of the following categories):

Experimental concept and design:	ca. 80%
Experimental work and/or acquisition of (experimental) data:	ca. 95%
Data analysis and interpretation:	ca. 75%
Preparation of figures and tables:	ca. 90%
Drafting of the manuscript:	ca. 75%

3.2 Publication I

Seasonal lipid dynamics of the shrimps *Crangon crangon* and *Pandalus montagui* in the German Bight (North Sea).

Diana Martínez-Alarcón, Reinhard Saborowski, Eleni Melis, Wilhelm Hagen

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Seasonal lipid dynamics of the shrimps *Crangon crangon* and *Pandalus montagui* in the German Bight (North Sea)

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ABSTRACT: Environmental fluctuations can impose energetic constraints on organisms in terms of food shortage or compensation for metabolic stress. To better understand the biochemical strategies that support adaptive physiological processes in variable environments, we studied the lipid dynamics of the brown shrimp *Crangon crangon* and the pink shrimp *Pandalus montagui* by analysing their midgut glands during an annual cycle. Both species have an overlapping distribution range in the southern North Sea, but differ in their habitat preferences, reproductive strategies, and life-history traits. *C. crangon* showed minor total lipid accumulation in their midgut glands, ranging between 14 and 17% of dry mass (DM), dominated by phospholipids. In contrast, *P. montagui* stored significantly larger amounts of total lipid (47–70% DM, mainly triacylglycerols) and showed a distinct seasonal cycle in lipid accumulation with a maximum in summer. Fatty acid trophic markers indicated a wide food spectrum for both species, with higher preferences of *P. montagui* for microalgae. In *C. crangon,* feeding preferences were less distinct due the low total lipid levels in the midgut gland. PCA based on fatty acid compositions of both species suggested that *C. crangon* has a broader dietary spectrum than *P. montagui*. *C. crangon* seems to have the capacity to use sufficient energy directly from ingested food to fuel all metabolic requirements, including multiple spawnings, without building up large lipid reserves in the midgut gland. *P. montagui*, in contrast, relies more on the energy storage function of the midgut gland to overcome food scarcity and to allocate lipids for reproduction.

KEY WORDS: Lipids · Fatty acids · Trophic markers · Life-history traits · Adaptation · Variable environment

1. INTRODUCTION

Species that inhabit a highly variable environment such as the North Sea must have evolved physiological and behavioural adaptations to overcome challenging conditions. Adaptations are species-specific and depend on different external and internal factors such as food availability and feeding habits, metabolic properties, and reproductive processes. High flexibility in the digestive physiology of crustaceans is essential for their ability to grow and reproduce, given the strong variation of food supply across seasons (Sánchez-Paz et al. 2006, Martínez-Alarcón et al. 2018).

The brown shrimp *Crangon crangon* (Linnaeus, 1758) is highly abundant and plays a key role in the North Sea ecosystem. It is the most important target of coastal fisheries in the southern North Sea, yielding €128 million in 2014 (STECF 2016). *C. crangon* is exposed to a pronounced seasonal temperature cycle (Campos et al. 2012), with winter temperatures from below 0 to 4°C, summer temperatures of 18 to 20°C, and occasionally even 30°C in tide pools of the Wadden Sea (Reiser et al. 2014). Growth rates are highly

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variable and depend, among other factors, on size and sex (Hufnagl & Temming 2011). *C. crangon* is a multiple spawner. Egg-carrying females are observed during a long reproductive season starting in November. Maximum numbers of gravid females occur in February/March followed by the main hatching period in April/May (Siegel et al. 2008). The brown shrimp can act as a structuring force of benthic shallow-water communities (Campos et al. 2012). It is omnivorous and feeds opportunistically on various small invertebrates, algae, and carrion (Plagmann 1939, Pihl & Rosenberg 1984, Siegenthaler et al. 2019).

The pink shrimp *Pandalus montagui* (Leach, 1814) has a narrower thermal tolerance window than the brown shrimp. Typical features of its distribution areas are lower temperatures (usually below 10°C) and constant salinities between 32 and 34. *P. montagui* appears in shallow coastal waters in spring and summer, but recedes to deeper waters in autumn and winter (Simpson et al. 1967, Stevenson & Pierce 1985). Mature males change sex to become secondary females after the second or third year (Simpson et al. 1967). *P. montagui* is a single spawner with a short spawning period between November and February. It feeds mainly on polychaetes, but crustaceans, foraminifers, hydroids, and fish remains are also frequently found in their stomachs (Simpson et al. 1967). Though abundant and ecologically relevant, the economic importance of *P. montagui* in the North Sea is negligible.

In shrimps, lipids are a major energy source usually deposited in the midgut gland. Lipids are involved in various key processes, including growth, moulting, and reproduction. Lipid droplets may also be accumulated in the cells of other tissues such as ovaries, and serve as energy stores (Lee & Walker 1995). These lipid reserves are mobilized during periods of food deprivation (Sánchez-Paz et al. 2006) and gonad maturation. During starvation, crustaceans preferentially catabolize neutral lipids, e.g. triacylglycerols, while polar lipids, mainly phospholipids, are conserved due to their important function as structural components of cell membranes (Heath & Barnes 1970, Stuck et al. 1996, Hervant et al. 1999). However, the relative importance of metabolic reserves and the intensity of their utilization vary among species (Hervant et al. 1999). *C. crangon* and *P. montagui* from the North Sea have been analysed for proteins and enzyme activities (Teschke & Saborowski 2005, Saborowski et al. 2012), as well as for lipid compositions of muscle of *C. crangon* (Mika et al. 2014) from the Gulf of Gdansk and of whole *P. montagui* (Clarke 1979a). According to Mika et al. (2014), the lipids in the abdominal muscle of *C. crangon* were mainly composed of neutral lipids, while Clarke (1979a) reported that the dominant lipid classes in *P. montagui* were polar lipids and triacylglycerols. In order to better understand the biochemical and physiological aspects of storage capacities, nutritional requirements, and finally, life strategies of these North Sea shrimps, it is necessary to analyse the lipid composition of their midgut gland, which is the main organ of lipid storage in decapod crustaceans.

Complementary to the investigations of Clarke (1979a) and Mika et al. (2014), we determined total lipid contents, lipid classes, and fatty acid (FA) compositions of the midgut glands of *C. crangon* and *P. montagui* from the North Sea. Feeding preferences were also determined by applying the FA trophic marker concept (Dalsgaard et al. 2003). Samples were collected in February, April, July, and October 2016 for seasonal coverage.

We hypothesized that the importance of lipid depots in the midgut gland differs between both species, and that lipid levels are linked to the reproductive cycle and the reproductive effort of either species. We also hypothesized that feeding preferences will vary between species among seasons.

2. MATERIALS AND METHODS

2.1. Collection of shrimp samples

Specimens of *Crangon crangon* and *Pandalus montagui* were collected by bottom trawling with the RV 'Uthörn' during 4 sampling campaigns. The sampling site was located in the southern North Sea near the island of Helgoland. Sampling was carried out in February, April, July, and October 2016 (Table 1). Directly after collection, adult specimens of similar size were sorted from the catch. Total body length (rostrum to tip of telson) and sex of the animals were determined on board. The midgut glands of the animals were dissected, shock-frozen in liquid nitrogen, transported to the Marine Zoology laboratory at the University of Bremen, and stored at −80°C until further analysis.

2.2. Total lipid, lipid class, and FA analyses

Dry mass (DM) of the midgut glands was determined after lyophilisation for 48 h (CHRIST Alpha 1-4 LD plus). Total lipids were extracted from the

Table 1. Sampling time, location, and biological data of *Crangon crangon* and *Pandalus montagui*. No specimens of *P. montagui* were available in February. SND: sex not determined

Date (2016)	Location	No. ind. analysed	Total length (mm)	Male (n)	Female (n)	Females without eggs (n)	Females with eggs (n)	SND (n)
C. crangon								
February 19	54°08′N, 07°52′E	20	56–68	0	19	3	16	1
April 18	53°44′N, 08°15′E	29	55–80	6	23	0	23	0
July 19–21	53°44′N, 08°15′E	14	55–68	7	7	0	7	0
October 24–26	54°08′N, 07°52′E	14	63–78	3	11	10	1	0
P. montagui								
April 18	53°44′N, 08°15′E	20	56–82	5	11	10	1	4
July 19–21	53°44′N, 08°15′E	15	59–72	6	8	8	0	1
October 24–26	54°08′N, 07°52′E	13	41–68	0	0	0	0	13

midgut gland samples after Folch et al. (1957) with dichloromethane:methanol (2:1 v/v) and an aqueous solution of KCl (0.88%). The amount of total lipids was determined gravimetrically after Hagen (2000) and expressed as the percentage of lipids in relation to the DM of the sample (total lipid in %DM).

Lipid class compositions of the midgut gland from individuals sampled in February, April, and October were analysed in triplicate and quantified by thin-layer chromatography–flame-ionisation detection (TLC-FID) on an Iatroscan Mark V device after Fraser et al. (1985). Calibration was done with single compound standards as listed in Table 2.

FAs were first converted to their methyl ester derivatives (FAMEs) by transesterification for 4 h at 80°C in hexane and methanol containing 3% concentrated sulphuric acid (Kattner & Fricke 1986). FAMEs were extracted with aqua bidest. (DDW) and hexane. After centrifugation, the upper lipid phase was transferred into a clean sample vial and the vial placed in an evaporator (N-EVAP model 112, Organomation) to dry the sample with a gentle stream of nitrogen. Subsequently, the samples were analysed by gas chromatography (Agilent Technologies, GC model 7890A). The device was equipped with a DB-FFAP column (30 m length, 0.25 mm inner diameter) and a programmable temperature vaporizer injector, operating with helium as the carrier gas. FAs were identified by their retention times in comparison to known FA standard compositions (FAMEs of the copepod *Calanus hyperboreus* and menhaden fish oil) (Schukat et al. 2014, Bode et al. 2015).

The FA compositions were evaluated according to the FA trophic marker concept of Dalsgaard et al. (2003), where 16:1(n-7), 16:4(n-1), and 18:1(n-7) are indicators of diatom-dominated food sources, and 18:4(n-3) is used as a dinoflagellate marker. Also, according to St. John & Lund (1996), Dalsgaard et al. (2003), and Bode et al. (2015), the ratio 16:1(n-7)/16:0 can be used as another index of diatom feeding (values >1 indicate significant feeding on diatoms). The carnivory:herbivory index (CHI) was determined after Schukat et al. (2014), as modified by Bode et al. (2015), by dividing the 18:1(n-9) content by the sum of all herbivorous biomarkers and 18:1(n-9), i.e. CHI = 18:1(n-9)/(16:1(n-7)+18:1(n-7)+18:1(n-9)). Free fatty alcohols and unidentified components with <1% of total FA concentrations were not considered for analysis.

Table 2. Standard compounds for lipid class calibration

Lipid class	Abbreviation	Standard	Sigma Aldrich art. no.
Phospholipid	PL	L-α-phosphatidylcholine	P3556
Sterol	ST	Cholesterol	C8667
Free fatty acid	FFA	Oleic acid	O1008
Diacylglycerol	DAG	Distearoylglycerol	D9019
Triacylglycerol	TAG	Glyceryltrioleate	T7140
Wax ester	WE	Laurylpalmitoleate	P1642

2.3. Statistical analysis

The FA data sets were arcsine-square-root transformed to establish normal distributions and homogeneity of variance. To test for differences in the total lipid contents and the FA concentrations between seasons, a 1-way ANOVA was used. Differences among groups were identified by

pairwise comparison with Tukey's post hoc test. The level for statistical significance was set at $\alpha = 0.05$. In order to identify species-specific differences in the FA compositions of *C. crangon* and *P. montagui,* a PCA was conducted. Males and females were pooled for the seasonal analyses after a Student's *t*-test showed no significant sex-related differences. Differences in the amount of total lipid (%DM) between species were also tested with Student's *t*-test. All statistical tests and graphic presentations were carried out with the RStudio software, version 0.99.491.

3. RESULTS

3.1. Total lipid contents and lipid class compositions

Throughout the year, the amount of total lipid (%DM) was significantly lower in the midgut glands of *Crangon crangon* than in those of *Pandalus montagui* ($p < 0.01$). The brown shrimps had mean total lipid levels between 14% DM in July and February and 17% DM in October. Total lipids in the midgut glands of the pink shrimp ranged from 47% DM in April to 70% DM in July (Fig. 1). In *C. crangon* midgut glands, the amount of total lipid (%DM) changed significantly from July to October and from October to February (Fig. 1a). In *P. montagui* midgut glands, the total lipid amount (% DM) increased significantly from April to July, and decreased significantly again towards October (Fig. 1b). No specimens of *P. montagui* were available in February, due to its seasonal migration to deeper waters. No statistical differences were detected between males and females of either species. The mean amount (± SD) of total lipid in the midgut glands of *C. crangon* was 14.7 ± 6.8% DM in females and 15.4 ± 5.6% DM in males ($p = 0.68$). In *P. montagui*, the mean amount of total lipid was 57.0 ± 14.6% DM in females and 56.9 ± 16.1% DM in males ($p = 0.99$).

The lipid class composition (% of total lipid, TL) of the midgut glands of *C. crangon* was dominated by phospholipids (PL: 75% TL), followed by triacylglycerols (TAGs: 17% TL) and cholesterol (Chol+DAG: 7% TL). In *P. montagui*, TAGs prevailed (73% TL), followed by PL (26% TL) and Chol+DAG (1% TL) (Table 3). No free FAs were detected, indicating that no autolytic degradation processes occurred during collection, dissection, and storage.

3.2. FA compositions

FA compositions of the midgut glands of both species are presented in Table 4. In *C. crangon*, principal FAs were 20:5(n-3), 22:6(n-3), 16:0, 18:1(n-9), and 16:1(n-7). The portion of polyunsaturated FAs (PUFAs) was higher (38–47% of total FAs) than that of monounsaturated (MUFAs, 26–31%) and saturated

Table 3. Lipid class compositions (mean ± SD % total lipid) in the midgut glands of *Crangon crangon* (n = 10) and *Pandalus montagui* (n = 8). Data sets include females and males collected in February, April, and October. TAG: triacylglycerols; PL: phospholipids; Chol: sterols; DAG: diacylglycerol; FFA: free fatty acids

Species	TAG	PL	Chol+DAG	FFA
C. crangon	17 ± 11	75 ± 14	7 ± 1	Below detection limit
P. montagui	73 ± 10	26 ± 12	1 ± 1	<1%

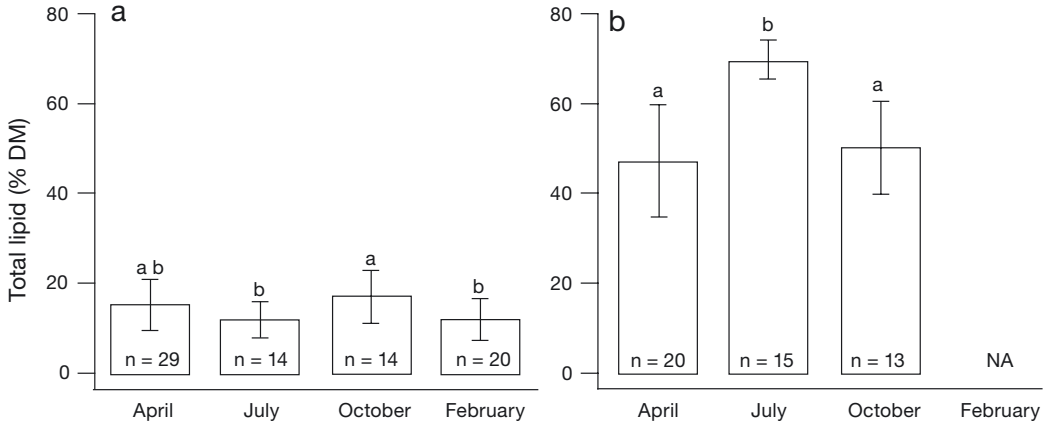

Fig. 1. Total lipid contents (% of dry mass, DM) of the midgut glands of (a) *Crangon crangon* and (b) *Pandalus montagui* from different seasons in 2016 (mean ± SD). Different letters above bars indicate significantly different values. NA: not available

FAs (SFAs, 18–23%). Palmitic acid (16:0) dominated within the SFAs. 16:1(n-7) and 18:1(n-9) prevailed in the MUFAs. Eicosapentaenoic acid (EPA, 20:5(n-3)) and docosahexaenoic acid (DHA, 22:6(n-3)) were the dominant components of the PUFAs.

In *P. montagui,* the dominant MUFAs were 16:1(n-7) and 18:1(n-9), while 20:5(n-3) and 22:6(n-3) prevailed in the PUFA fraction. In April, percentages of MUFAs were higher than those of the PUFAs. In July and October, the portion of PUFAs exceeded that of MUFAs. In all cases, SFAs comprised the smallest fraction.

PCA based on FA compositions of the midgut gland of *C. crangon* grouped the different months in unspecific overlapping clusters (Fig. 2a). Two principal components (PCs) explained 58% of the variance. The first PC was mainly represented by positive values of the FAs 18:1(n-9) and 20:4(n-6), and negative values of 14:0 and 20:1(n-11). The second PC was mostly characterized by positive values of the FAs 20:1(n-7) and 16:3(n-4), and negative values of 18:0 and 18:1(n-7). In contrast, PCA on the FA compositions of the midgut gland of *P. montagui* grouped the different months in specific clusters (Fig. 2b). Two main PCs explained 67% of the variance. The main contributing vectors of PC1 were 18:1(n-7) and 18:0 with positive values, and 14:0 with a negative value. PC2 was mostly defined by 20:1(n-7) and negative values of 20:4(n-6).

Combined PCA of both species in April (Fig. 3a), July (Fig. 3b), and October (Fig. 3c) showed more clearly defined clusters for *P. montagui* than for *C. crangon*. The clusters overlapped in July, but not in April and only very little in October. Two PCs explained 55% of the variance in April, 69% in July, and 58% in October. In the 3 PCAs, the vector of 20:4(n-6) appeared to have an impact on the *C. crangon* clusters, while the 14:0 vector was more directed towards *P. montagui*. The vector 18:1(n-9) was directed towards *P. montagui* in April, but towards *C. crangon* in July and October. Finally, the vector 16:1(n-7) contributed to PC1 with negative values in April and July in the direction of the *P. montagui* clusters.

4. DISCUSSION

The southern North Sea at 54° N is subjected to distinct seasonal changes in irradiation and temperature (Otto et al. 1990, van Aken 2008, Sündermann & Pohlmann 2011, Neumann et al. 2017). Productivity and biomass of the system vary concordantly (Beu-

Table 4. Fatty acid compositions of *Crangon crangon* and *Pandalus montagui* expressed as percentage of total fatty acids (% TFA). Sum of herbivory markers (%) (16:1(n-7) and 18:1(n-7)), diatom ratio (16:1(n-7)/16:0), and carnivory:herbivory index (18:1(n-9)/ herb.+18:1(n-9)) are also presented. Values are given as means ± SD. n: number of samples analysed. Concentrations <1% TFA are not presented

Species	*C. crangon*				*P. montagui*		
	April (n = 15)	July (n = 14)	October (n = 12)	February (n = 20)	April (n = 19)	July (n = 15)	October (n = 13)
Fatty acids (%)							
14:0	2.5 ± 0.9	1.9 ± 1.2	3.2 ± 1.3	1.1 ± 0.6	3.1 ± 0.7	3.9 ± 1.3	3.9 ± 0.4
16:0	14.6 ± 1.4	14.7 ± 3.2	14.1 ± 2.7	12.9 ± 1.5	14.1 ± 1.4	14.1 ± 0.8	14.5 ± 1.5
16:1(n-7)	9.7 ± 3.2	6.8 ± 3.7	10.4 ± 4.0	6.3 ± 2.5	14.1 ± 2.3	12.2 ± 4.0	11.3 ± 1.8
iso 17:0	1.4 ± 0.3	1.9 ± 1.5	1.6 ± 0.7	1.4 ± 0.4	1.6 ± 0.5	1.4 ± 0.2	1.0 ± 0.2
16:3(n-4)	0.9 ± 0.1	1.1 ± 0.4	1.3 ± 0.3	1.8 ± 1.5	1.1 ± 0.1	0.9 ± 0.2	1.0 ± 0.1
18:0	3.9 ± 1.0	5.1 ± 1.4	3.6 ± 0.6	3.1 ± 0.5	3.8 ± 0.3	4.3 ± 1.2	3.6 ± 0.3
18:1(n-9)	8.8 ± 1.7	11.2 ± 2.9	11.7 ± 1.0	12.2 ± 1.6	10.1 ± 1.1	8.9 ± 1.7	9.5 ± 0.6
18:1(n-7)	5.0 ± 0.8	5.5 ± 0.7	5.4 ± 0.8	6.6 ± 0.9	5.9 ± 0.5	6.3 ± 0.9	5.3 ± 0.6
20:1(n-11)	0.9 ± 0.4	1.5 ± 1.5	1.6 ± 0.8	1.5 ± 0.9	2.5 ± 0.8	2.3 ± 0.8	1.2 ± 0.6
20:1(n-7)	2.3 ± 0.9	0.9 ± 0.8	2.2 ± 1.1	1.4 ± 1.0	2.0 ± 0.5	1.8 ± 0.3	1.3 ± 0.3
20:4(n-6)	3.1 ± 0.7	4.0 ± 1.4	3.6 ± 1.0	4.5 ± 1.2	1.8 ± 0.3	2.5 ± 1.2	2.1 ± 0.2
20:5(n-3)	20.3 ± 3.8	19.1 ± 4.4	16.2 ± 4.3	19.9 ± 2.8	15.6 ± 2.6	18.1 ± 2.3	18.3 ± 1.5
22:5(n-3)	2.6 ± 0.9	3.3 ± 2.7	2.1 ± 1.3	2.7 ± 1.5	2.6 ± 1.2	2.8 ± 0.6	1.5 ± 0.3
22:6(n-3)	15.7 ± 3.1	17.0 ± 4.7	14.9 ± 4.5	18.7 ± 3.2	11.1 ± 1.4	10.4 ± 2.9	13.4 ± 1.2
Σ saturated	22.4 ± 1.1	23.5 ± 0.5	22.5 ± 0.8	18.4 ± 1.3	22.6 ± 1.4	23.7 ± 1.1	23.0 ± 1.7
Σ monounsaturated	26.7 ± 0.7	25.9 ± 0.5	31.4 ± 0.6	28.0 ± 0.7	34.6 ± 1.0	31.5 ± 0.7	28.6 ± 1.2
Σ polyunsaturated	42.6 ± 0.6	44.5 ± 0.4	38.0 ± 0.4	47.6 ± 0.5	32.2 ± 0.9	34.7 ± 0.7	36.3 ± 1.5
Sum of herbivory markers (%)	14.7 ± 3.1	12.3 ± 3.9	15.8 ± 4.2	12.9 ± 2.6	19.9 ± 2.3	18.4 ± 3.9	16.6 ± 1.8
Diatom ratio	0.7 ± 0.2	0.5 ± 0.3	0.7 ± 0.3	0.5 ± 0.2	1.0 ± 0.1	0.9 ± 0.3	0.8 ± 0.1
Carnivory:herbivory index	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.3 ± 0.0	0.3 ± 0.1	0.4 ± 0.0

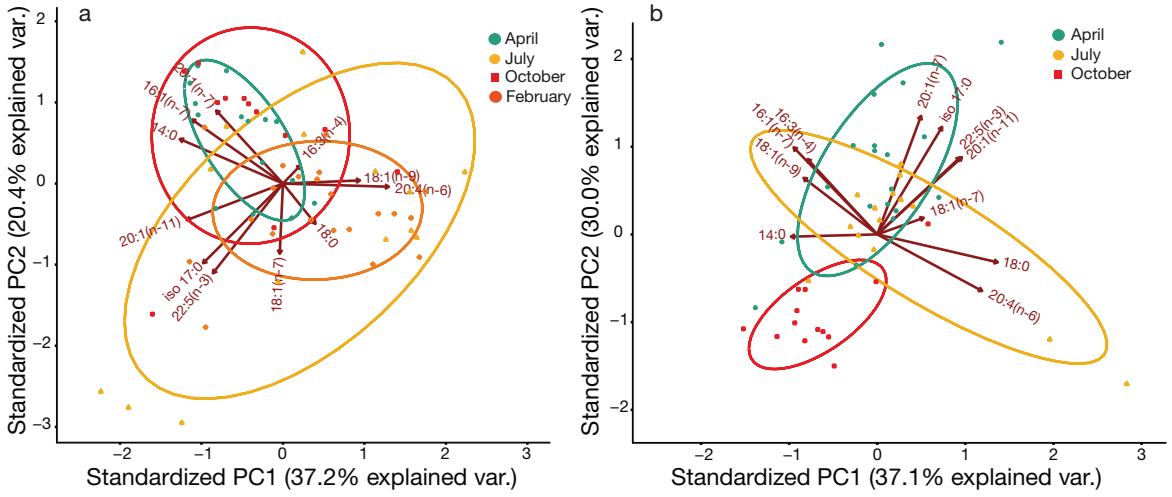

Fig. 2. PCA based on seasonal fatty acid compositions of the midgut glands of (a) *Crangon crangon* and (b) *Pandalus montagui*. The ellipses group data at a confidence level of 95%

kema 1974, Reiss & Kröncke 2004). The roughly bimodal phytoplankton bloom occurs from March to June and ends in autumn (Wiltshire et al. 2015), with secondary production rising in between (Roff et al. 1988). Therefore, it can be expected that lipid dynamics follow a seasonal pattern in both shrimp species, in accordance with seasonal environmental factors and to buffer food availability.

Although both species share the same habitat, they show pronounced differences in their lipid storage strategies. *Pandalus montagui* accumulated significantly higher amounts of total lipids in the midgut gland, and showed a more distinct seasonal lipid variation than *Crangon crangon*. Differences between species were also reflected in their lipid class compositions. *P. montagui* accumulated TAGs in the midgut gland, which are typical storage lipids (Lee et al. 2006), whereas the midgut glands of *C. crangon* contained very little TAG. Due to this low lipid level, *C. crangon* had high percentages of phospholipids, which are essential structural components of biomembranes rather than storage lipids. Hence, lipid accumulation in *C. crangon* was low during summer, and increased only slightly in spring and autumn. These results are consistent with other previous measurements in our laboratory: total lipid contents of midgut glands of *C. crangon* from the southern North Sea ranged between 13% DM in March and 20% DM in July (C. Sahlmann unpubl. data) and between 10% DM in May and 32% DM in September (K. Pöhlmann unpubl. data), which indicates some interannual variability. In contrast, lipid levels in the midgut glands of *P. montagui* increased significantly during the productive summer season, from 47% to a maximum of 70% DM, indicating intensive lipid storage activity.

4.1. Lipid deposition

The energy density of lipids is twice as high as that of carbohydrates and proteins. Therefore, accumulation of energy reserves via lipid stores, predominantly TAGs and wax esters (WEs), is the most efficient and most common means of energy storage in marine invertebrates, especially herbivorous zooplankton. The stores are used to overcome periods of food paucity or for the transfer of energy towards reproductive processes (Lee et al. 2006). The midgut gland of crustaceans is generally accepted as the central metabolic organ and the principal lipid storage site (O'Connor & Gilbert 1968). Other arthropods, e.g. insects, possess a lipid storage organ called the fat body (Arrese & Soulages 2010), which is considered to be unique to this taxon of arthropods (Law & Wells 1989). Several studies, however, reported the involvement of a fat body in the vitellogenesis of crustaceans such as isopods (Picaud 1980, Souty & Picaud 1981) and euphausiids (Cuzin-Roudy 1993). However, the presence of an explicit fat body has not been confirmed for decapods.

In addition to the midgut gland, muscle tissue may also act as a lipid storage organ. Mika et al. (2014) reported that the muscle of *C. crangon* contained 32.2 mg total lipid g^{-1} wet mass in spring and 7.7 mg

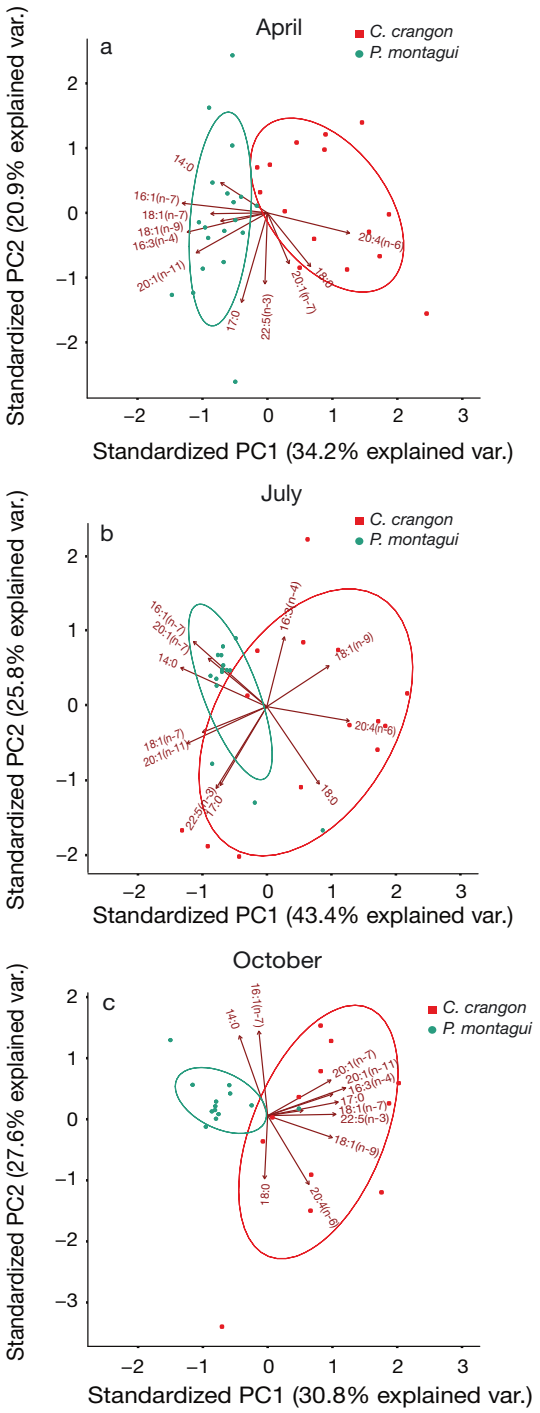

Fig. 3. PCA based on the fatty acid compositions of midgut glands of *Crangon crangon* and *Pandalus montagui* in (a) April, (b) July, and (c) October. The ellipses group data at a confidence level of 95%

total lipid g^{-1} wet mass in summer. This corresponds to 128.8 mg (12.9% DM) and 30.8 mg (3.1% DM) on a dry mass basis assuming a water content of 75%. These values are in the same range or clearly below the lipid contents we determined in the midgut gland. Therefore, it is unlikely that muscle tissue represents an important storage organ for lipids in *C. crangon*.

4.2. Food and trophic markers

C. crangon is an opportunistic omnivorous predator on micro-, meso-, and macrofauna. Smaller specimens feed on ostracods and harpacticoid copepods, while larger shrimps prefer various species of mussels, polychaetes, and small crustaceans (Plagmann 1939, Pihl & Rosenberg 1984, Feller 2006). Cannibalism is also common (Pihl & Rosenberg 1984). Microphytoplankton, such as diatoms and dinoflagellates, are ingested when they become abundant during the seasonal plankton blooms. Additionally, macroalgae of the genus *Ulva* and *Enteromorpha* were found in the stomachs of *C. crangon*. During the course of the year the shrimps show a pronounced trophic flexibility, and change their dietary spectrum according to the availability of the food organisms (Plagmann 1939). The preferred food of *P. montagui* covers a similar spectrum to that of *C. crangon*. It consists of polychaetes, crustaceans, and other small pelagic and benthic organisms (Simpson et al. 1967).

According to the FA trophic marker concept, ingested marker FAs are incorporated unmodified, usually in lipid depots, and can provide information about the trophic preferences of species (Dalsgaard et al. 2003). *C. crangon* showed low total lipid levels and, consequently, a higher portion of polar lipids. Moreover, PUFAs are more frequent as important components of biomembranes in the polar lipid fraction (Jezyk & Penicnak 1966), which explains the larger fraction of PUFAs in the midgut gland of *C. crangon* compared to *P. montagui* (Table 4).

Diatoms and dinoflagellates differ in their FA compositions. Typical FAs of diatoms are 16:1(n-7), 18:1(n-7), 20:5(n-3), and C16 PUFAs. Dinoflagellates are rich in 18:4(n-3) and 22:6(n-3) (Graeve et al. 1994). Due to the prevalence of neutral lipids in the pink shrimp, the concentrations of those FAs and the higher ratio 16:1(n-7)/16:0 strongly suggest that *P. montagui* feed on diatoms and dinoflagellates, but in different seasons. The carnivory marker 18:1(n-9) was higher in April than in the other months, while the CHI remained at the same level among seasons,

indicating a wide food spectrum and an omnivorous feeding behaviour.

For *C. crangon*, such assumptions are less validated due to the low total lipid content, and thus high phospholipid fraction. The concentrations of 16:1(n-7) indicate that *C. crangon* feed on diatoms, and high levels of 16:1(n-7) in April match with the phytoplankton spring bloom (Wiltshire et al. 2015). The variation in the 16:1(n-7)/16:0 ratio, which also indicates diatom feeding, followed the same pattern as the total lipid amount in the midgut gland of *C. crangon*. Therefore, it may be possible that the low lipid level in the midgut gland of *C. crangon* partly originates from diatoms. Elevated levels of 22:6(n-3) in July coincide with a high dinoflagellate abundance (Löder et al. 2012). However, there is not enough evidence to state that they feed significantly on dinoflagellates, because the FA 18:4(n-3), another relevant dinoflagellate marker, accounts for <1% of total FAs. As the FA 22:6(n-3) is one of the principal (non-dietary) components of the lipid membrane, the inverse relationship with 16:1(n-7) could also point to a higher portion of this FA in the membrane lipids. Accordingly, at relatively high lipid levels in April and October, the percentage of 22:6(n-3) decreased, but increased in July and February, when lipid levels were low.

PCA based on the FA composition of *C. crangon* was significantly affected by the membrane FAs. Therefore, we excluded those FAs from this analysis to emphasize the trophic markers. No clustering of FAs was evident between months in *C. crangon,* which indicates that this species did not display different seasonal feeding preferences in our study. *P. montagui* showed 3 distinct clusters corresponding to the 3 sampling months, which suggests a distinct seasonal change of food preferences by *P. montagui* (Fig. 2). Diatom markers mainly affected the clusters of April and July but not October (Fig. 3), which indicates that in April and July, *P. montagui* fed preferably on diatoms. The very well defined PCA clusters of *P. montagui,* especially in July and October, compared to the broader clusters of *C. crangon*, suggest more specific feeding preferences of *P. montagui* and a broader dietary spectrum of *C. crangon* (Fig. 3).

4.3. Lipids and reproduction

Both species, *C. crangon* and *P. montagui*, are r-strategists showing fast growth, short longevity, high fecundity, and small but numerous eggs. They exhibit a higher individual annual reproductive effort than K-strategists (slow growth, deferred maturity, greater longevity, low fecundity, large yolky eggs) (Clarke 1979b). When food availability for planktonic offspring is patchy or unpredictable, it is less risky and more efficient to produce many small eggs (Vance 1973, Clarke 1979b). Due to the variable environmental conditions of the North Sea, species following the r-strategy will probably be more successful.

C. crangon show intensive reproductive activities in the southern North Sea. Egg-bearing females of *C. crangon* are present almost year-round, but mainly from November to September, with the number of ovigerous females peaking in spring and early summer (Boddeke & Becker 1979, Siegel et al. 2008, Campos et al. 2010). Females can repeatedly produce eggs, and the clutch size varies from 1000 to 14 000 eggs (Clarke 1979b). In the German Bight, they may spawn 2 to 3 times during the season (Ehrenbaum 1890, Havinga 1930). Meixner (1966) even reported up to 5 spawnings in an aquarium at 14°C water temperature.

Egg-carrying females of *P. montagui* occur largely between November and March, and planktonic larvae are released only once per year (Allen 1963), mainly during spring (Simpson et al. 1967). During our study, no females with eggs were caught, since sampling took place outside the spawning season. According to Clarke (1979a), ovigerous females of *P. montagui* have low lipid contents in the midgut glands, whereas midgut glands of females with maturing ovaries have high lipid levels. Therefore, the elevated lipid contents in the midgut glands found in July may reflect the presence of females in the early phase of maturing ovaries (Fig. 1). Our results agree with the observations of Warren (1973) who stated that ovary development in *P. montagui* is paralleled by a decrease in total lipids in the midgut gland. Apparently, from July to October the shrimps transfer lipids from the midgut gland to the maturing ovaries to prepare for the upcoming spawning season between November and March.

The eggs of *C. crangon* contain almost 60% DM protein and 33% DM lipid (Pandian 1967). Since their eggs are smaller than those of *P. montagui*, they contain less lipid (5.3 µg vs. 23.2 µg egg^{-1}). However, the clutch size is much higher in *C. crangon* (1000–14 000 vs. 150–4000 in *P. montagui*; Clarke 1979b). Still, the total amount of lipid would be lower for an average egg clutch of *C. crangon* (39.8 mg) compared to that of *P. montagui* (48.1 mg).

C. crangon, the multiple spawner, exhibited low lipid-storage capacities, whereas *P. montagui,* the

single spawner, deposited much higher amounts of lipid in the midgut gland. The opposite was observed in the brachyuran crabs *Carcinus maenas* and *Hemigrapsus sanguineus* (Jungblut et al. 2018). *C. maenas,* a single spawner, has low lipid reserves, whereas *H. sanguineus,* which spawn several times a year, have high lipid levels and a strong seasonal variation in lipid levels (Fig. 4). These contrasting results suggest that lipid levels may not be related to the frequency of spawning. Apparently, the differences in lipid deposition in *C. crangon* and *P. montagui* may be explained by their deviating strategies to use the midgut gland for metabolic activities and for storage (Fig. 5). Similar to *C. crangon*, the midgut gland of the Antarctic shrimp *Chorismus antarcticus* acts as an active metabolic centre rather than a lipid storage organ (Clarke 1982). The metabolic activity and efficiency of the midgut gland allow immediate processing of dietary lipids, and reduce the need for large lipid stores in the midgut gland. This energetic strategy, however, demands continuous food supply and specific physiological and biochemical adaptations (Martínez-Alarcón et al. 2019), at least during the extended period of gonad maturation. Apparently, *C. crangon* is usually not confronted with prolonged periods of food deprivation, but may be adapted to cope at least with shorter starvation periods, e.g. by reducing metabolic rates (T. Werner pers. comm.).

This study also showed no statistically significant differences in total lipid contents of the midgut glands between sexes in both species. Similar observations were reported for the crabs *Armases cinereum* and *Sesarma* nr. *reticulatum* (Hasek & Felder 2005), *Aegla platensis* (Oliveira et al. 2007), the crayfish *Parastacus defossus* (Buckup et al. 2008), the green shore crab *Carcinus maenas,* and the Asian shore crab *Hemigrapsus sanguineus* (Jungblut et al.

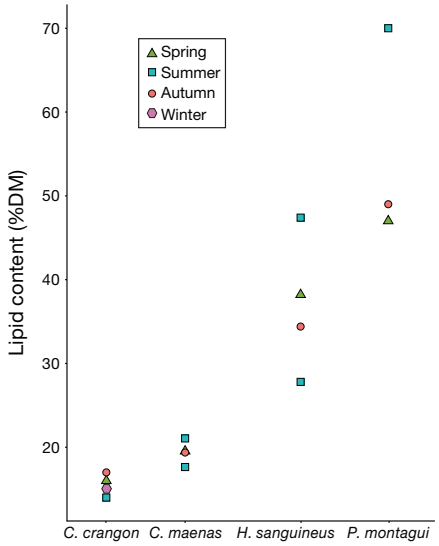

Fig. 4. Seasonal variation in mean lipid content in the midgut glands of *Carcinus maenas, Crangon crangon*, *Hemigrapsus sanguineus*, and *Pandalus montagui.* Data for *C. maenas* and *H. sanguineus* from Jungblut et al. (2018)

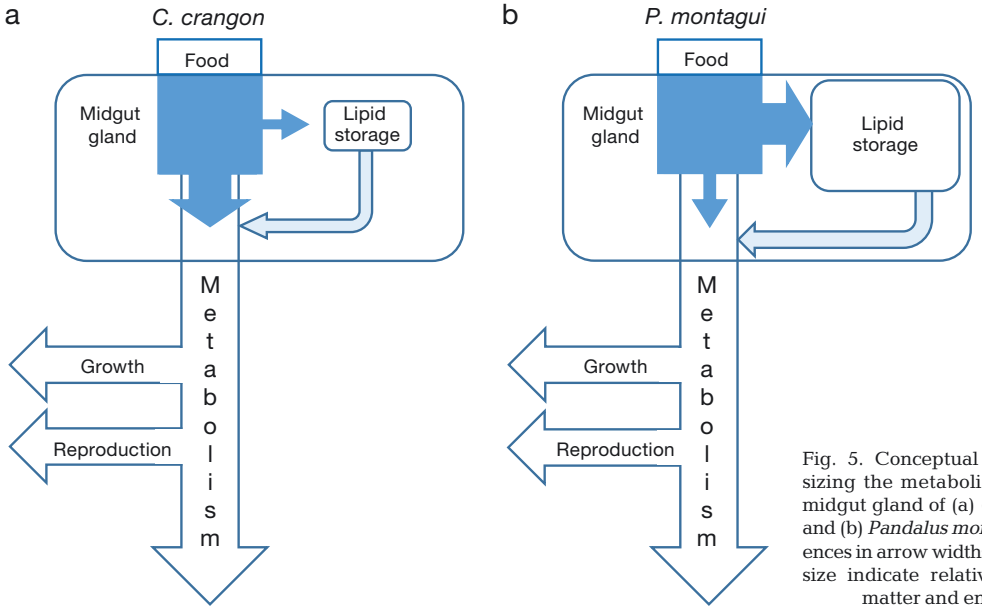

Fig. 5. Conceptual scheme emphasizing the metabolic function of the midgut gland of (a) *Crangon crangon* and (b) *Pandalus montagui.* The differences in arrow widths and lipid storage size indicate relative differences in matter and energy flows

2018). This is surprising, since a significant transfer of lipids from the midgut gland to the ovaries should result in pronounced differences between the sexes, as the energetic costs for the production of sperm is much lower compared to the egg production of females (Hayward & Gillooly 2011). Our findings also indicate that the ability of lipid storage is an intrinsic, probably genetically determined trait, at least in the species listed here. Further studies are required to elucidate the underlying biochemical processes in lipid metabolism and lipid-storage capacities in decapod crustaceans, also with regard to the different sexes.

We conclude that *C. crangon* and *P. montagui* follow very different energetic strategies in the southern North Sea, possibly related to the more northern distribution centre of the latter, which also tends to inhabit deeper water layers. In *P. montagui*, the variable but usually high lipid levels of the midgut gland are, to a certain extent, linked to the reproductive cycle. Lipid levels change according to reproductive seasons, but they are not sex-specific. Feeding preferences in the omnivorous *P. montagui* also vary with the seasons. In the omnivorous *C. crangon,* the low lipid content indicates that the midgut gland does not function primarily as an energy depot to support reproductive processes. Apparently, the midgut gland in *C. crangon* rather serves as a dynamic metabolic centre with high turnover rates. Hence, in spite of the limited lipid-storage capacity, the brown shrimp is well adapted to cope with a highly variable environment with periods of food paucity, and it has successfully established large stocks in the North Sea and surrounding waters.

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3.3 Publication II

Transcriptome analysis of the midgut gland of the brown shrimp *Crangon crangon* indicates high polymorphism in digestive enzymes.

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Transcriptome analysis of the midgut gland of the brown shrimp *Crangon crangon* indicates high polymorphism in digestive enzymes

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ABSTRACT

Tolerance of organisms towards heterogeneous and variable environments is highly related to physiological flexibility. An effective strategy to enhance physiological flexibility is the expression of polymorphic enzymes. This seems to be the case in the brown shrimp *Crangon crangon*. It shows high reproduction rates, feeds opportunistically on endo- and epibenthic organisms, and is apparently well adapted to variable environmental conditions. Previous electrophoretic studies revealed a high level of polymorphism and no consistent phenotype of digestive enzymes between individuals. In order to understand the underlying biochemical processes, we carried out a transcriptome-based study of digestive enzymes of *C. crangon*. Detailed sequence analyses of triacylglycerol lipase, phospholipase A_2, alpha amylase, chitinase, trypsin and cathepsin L were performed to identify putative isoforms. The number of isoforms, and thus the degree of polymorphism varied among enzymes: lipases and carbohydrases showed higher numbers of isoforms in enzymes that besides their extracellular function also have diverse intracellular functions. Furthermore, cysteine proteinases showed a lower polymorphism than serine proteinases. We suggest that the expression of enzyme isoforms improves the efficiency of *C. crangon* in gaining energy from different food sources.

1. Introduction

Evolution of proteins, control of gene expression, and metabolic regulation are just some examples of adaptive biological processes that enable organisms to tolerate the wide and variable spectrum of environmental conditions. In this respect, the expression of polymorphic enzymes has also been considered an effective strategy (Tomaiuolo et al., 2008). Understanding the physiological and evolutionary role of enzyme polymorphism has been the goal of biologists since several decades. Gillespie and Kojima (1968) and Kojima et al. (1970) reported a high variability of enzymes, which act on external substrates, so-called group II enzymes. The authors suggested that this variability reflects the pronounced structural variety of the respective substrates. Likewise, Johnson (1973, 1974) observed in several *Drosophila* species that enzymes, which degraded substrates from the external environment, were more variable than those, which processed substrates of internal metabolic pathways, the latter denoted as group I enzymes (Gillespie and Kojima, 1968). These findings seem reasonable, since changes in enzymes involved in the regulation of internal pathways would produce more pronounced alterations in the fitness of the organism than changes in enzymes, which act on external substrates.

Most research on enzyme polymorphism was done on enzyme loci in *Drosophila* (reviewed by Kumar and Singh, 2014). However, the study of enzyme loci requires genome-wide knowledge of the species of interest. Today, next-generation sequencing technologies allow us to study the relative abundance and variability of transcripts in any organism, also in species with scarce genetic information but with an extraordinary level of enzyme polymorphism. This is the case in the brown shrimp *Crangon crangon* (Linnaeus, 1758), which shows no consistent phenotype in digestive endopeptidases (Teschke and Saborowski, 2005; Saborowski et al., 2012). This variability among individuals even from the same population is not related to feeding habits. Another characteristic of *C. crangon,* which separates it from many other crustacean taxa, is the preferential expression of cysteine proteinases instead of serine proteinases to facilitate the degradation of dietary protein (Teschke and Saborowski, 2005).

Apparently, the brown shrimp is well adapted to thrive in a highly variable environment. It is an opportunistic feeder, shows high reproduction rates, and it is highly abundant in shallow coastal areas. Hence, it represents an important component of the North Sea ecosystem and plays a key role as predator and as prey. However, it is still unknown, how this species manages to cope with the pronounced

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environmental changes of the North Sea at the metabolic level. We hypothesise that part of this success is due to the polymorphism of digestive enzymes. Following this notion, we analysed the transcriptome of the midgut gland of *C. crangon* and used it as a basis for enzyme polymorphism studies. The main goal of this investigation is to assess the variability of selected digestive enzymes through putative isoforms and to better understand the underlying principles that trigger enzyme polymorphism in *C. crangon*. This study will also contribute to the understanding of adaptive molecular mechanisms that allow the brown shrimp to thrive in a very variable environment.

2. Materials and methods

2.1. Origin of samples

Specimens of the brown shrimp (*Crangon crangon*, Linnaeus, 1758) were collected on June 26th 2015 in the Weser estuary (North Sea, 53°48′N, 8°10′E) by bottom trawling with the research vessel FK *Uthörn*. Adult animals (> 55 mm total length) were randomly taken from the catch and transported to the laboratories of the Alfred Wegener Institute for Polar and Marine Research in Bremerhaven for further processing. The midgut glands were immediately dissected and transferred into 1.5 ml tubes containing 0.5 ml RNAlater. The samples were left overnight at room temperature to absorb the RNAlater solution and thereafter stored at −80 °C until RNA extraction. All applicable European and German guidelines for the use of marine invertebrate animals were followed in this study.

2.2. Total RNA isolation

Midgut gland samples stored at −80 °C in RNAlater were thawed and about 30 mg of tissue were removed for RNA extraction. Cell lysis was performed in 0.6 ml lysis buffer RLT (Qiagen, Hilden, Germany) with the Precellys keramik kit 1.4 mm (PEQLAB 91-PCS-CKM, Erlangen, Germany) using three cycles of 15 s shaking with 30 s pauses in between. Subsequently, samples were centrifuged at 13,000 × *g* for 3 min at room temperature. Total RNA was isolated using RNeasy Mini Kit spin columns (Qiagen 74104, Hilden, Germany) following the manufacturer's instructions. RNA quantities and purities were determined with a NanoDrop ND-1000 device at 260 nm and 260/280 nm, respectively. The quality of the RNA was analysed by microfluidic electrophoresis in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA).

2.3. Construction of normalized cDNA libraries and Illumina sequencing

Two separate pools of total RNA were prepared containing RNA from ten animals each. The construction of the normalized cDNA libraries was done by VERTIS Biotechnologie AG (Freising, Germany). First-strand cDNA synthesis was primed with a N6 randomized primer. After fragmentation the Illumina TruSeq sequencing adapters were ligated in a strand-specific manner to the 5′and 3′ ends of the cDNA fragments. Subsequently, sequencing of the cDNA libraries was performed on an Illumina MiSeq sequencer at the Alfred Wegener Institute.

2.4. De-novo assembly and functional annotation

Raw reads were quality-filtered using the software Trimmomatic in paired-end mode version 0.32 (Bolger et al., 2014). The following quality-filtering parameters were applied: leading and trailing quality of 3, a sliding window size of 5 bases with an average quality of 20 and a minimum length of 50 bases. The final quality of filtered datasets was checked using fastQC (version 0.10.01 – http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The quality scores of all reads were above 30. The sequencing data of both cDNA libraries were combined and *de novo* assembled using the Trinity genome-independent transcriptome assembler version 2.2.0 (Grabherr et al., 2011) with a minimum contig length of 200 bases. Functional annotation was performed using the Trinotate functional annotation suite version 3.0.1 (Grabherr et al., 2011).

2.5. Sequence analysis

After the annotation step all sequences identified as triacylglycerol lipase (EC 3.1.1.3), phospholipase A_2 (EC 3.1.1.4), alpha amylase (EC 3.2.1.1), chitinase (EC 3.2.1.14), trypsin (EC 3.4.21.4), and cathepsin L (EC 3.4.22.15) were selected. The transcripts of each enzyme were grouped and aligned. Isoforms were first selected by annotation and only sequences with high identity value were kept. The sequence data have been submitted to the GenBank database under the following accession numbers: triacylglycerol lipases (MH055763-MH055776), phospholipase A_2 (MH055777-MH055796), alpha amylase (MH055751-MH055762), chitinase (MH069216-MH069295), trypsin (MH035874-MH035882, MH069215), cathepsin L (MH069296-MH069494). According to the results of the alignments, the frequency of isoforms in the transcriptome was represented as pie charts for each of the analysed enzymes. Cladograms based on the amino acid sequences were constructed for each of the enzymes using the software CLC Genomics Workbench (Qiagen, Aarhus A/S, version 8.5.1). Neighbor-joining algorithms were used based on the Jukes and Cantor (1969). A bootstrap method based on 10,000 permutations was applied for the confidence analysis. Similarities of isoforms with specific groups of enzymes were determined by BLASTing all the sequences against the Genbank.

3. Results

3.1. Transcriptome sequencing and assembly

A total of 11.3 million and 13.5 million sequences were obtained from sample A and sample B, respectively. After quality filtering, 10.0 respectively 11.8 million reads were used for *de novo* assembly. The assembly results are summarized in Table 1.

3.2. Sequence annotation

Annotation was performed against different databases (Table 2). For the 136,016 transcripts, the homology search against the Uniprot Swiss-Prot and TrEMBL databases resulted in 32,619 (24%) and 47,149 (34.7%) hits, respectively (e-value < $1e^{-9}$). Homology search using the translated amino acid sequences resulted in 24,385 and 30,222 hits. Additionally, searches against the Pfam, signalP, TmHMM and eggNOG databases were performed and GOterms retrieved from the associated blast hits by mapping (Table 2).

3.3. Identification of selected digestive enzyme transcripts and putative isoforms

We identified transcripts of enzymes for lipid, carbohydrate, and

Table 1
Basic statistics of the *de novo* assembled transcriptome.

Features	Values
Number of genes	56,247
Number of transcripts	136,016
Longest transcript	14,702
Average transcript length	569
Median of transcript length	339
Shortest transcript	201
N50	682
N75	346

Table 2
General information about the functional annotation of the *de novo* assembly.

Database	No. of hits
Swiss-Prot-Uniprot (blastx)	32,619
TrEMBL-Uniprot (blastx)	47,149
Swiss-Prot-Uniprot (blastp)	24,385
TrEMBL-Uniprot (blastp)	30,222
Pfam	23,362
SignalP	2371
TmHMM	4712
eggNOG	13,489
GO-terms	31,485

Table 3
Selected transcripts involved in digestive metabolism and putative isoforms.

Category	Gene	Number of transcripts	Putative number of isoforms
Lipid metabolism	Triacylglycerol lipase	14	6
	Phospholipase A_2	20	12
Carbohydrate metabolism	Alpha amylase	12	4
	Chitinase	80	13
Protein metabolism	Trypsin	10	8
	Cathepsin L	199	12

protein digestion. We selected enzymes relevant for our study of *C. crangon* as well as enzymes that were previously reported to play an important role in digestive processes of other decapods. Regarding the enzymes involved in lipid metabolism we analysed triacylglycerol lipase and phospholipase A_2. For enzymes involved in carbohydrate metabolism, we studied alpha amylase and chitinase. Among the enzymes involved in protein metabolism we analysed trypsin and cathepsin L. Numbers of transcripts and putative isoforms are summarized in Table 3. However, we cannot exclude the possibility that some transcripts of enzymes, especially those induced under particular circumstances, may not have been expressed in the analysed individuals.

3.4. Putative isoforms with alternative transcripts

The translated amino acid partial sequences of the selected transcript sequences were aligned to verify putative isoforms. After alignment, cladograms were constructed to visualise the similarity between sequences. Pie charts complement the cladograms to visualise the transcript count for each isoform and the number of identified isoforms. Each colour in the pie charts represents a different isoform. Among lipases, the major part of triacylglycerol lipase transcripts corresponds to two main isoforms (Fig. 1a). Phospholipase A_2, in contrast, does not show a particular isoform preference (Fig. 1b). Within the carbohydrate category, the majority of the alpha amylase sequences were allocated to two isoforms (Fig. 1c), while chitinase had at least four main isoforms (Fig. 1d). The serine protease trypsin showed a highly diverse cladogram. The majority of the isoforms had no high recurrence (Fig. 1e). Finally, the cysteine protease cathepsin L showed mainly four isoforms (Fig. 1f). The enzymes with most recurrent isoforms are triacylglycerol lipase, alpha amylase, and cathepsin L (Table 3).

3.5. Similarity in the sequences of C. crangon and the database

The best matches found in the database of the National Center of Biotechnology Information (NCBI) were chosen for each of the putative isoforms. Phospholipase A_2 and chitinase showed the most variable hits, whereas alpha amylase and trypsin were least variable (Table 4).

4. Discussion

Transcriptome data of the hepatopancreas of *Crangon crangon* were analysed to determine the degree of polymorphism of selected digestive enzymes. We focused our study on enzymes involved in lipid metabolism (triacylglycerol lipase, phospholipase A_2), carbohydrate digestion (chitinase, alpha amylase) and protein utilization (trypsin, cathepsin L).

4.1. Enzyme polymorphism

Kimura and Ohta (1971) described protein polymorphism as a phase of molecular evolution. It represents the first step in the long-lasting establishment of mutations within populations. Enzyme polymorphism has been predominantly studied in *Drosophila* (*e.g.*Van Delden et al., 1978; Kumar and Singh, 2014; Chakraborty and Fry, 2016). Tomaiuolo et al. (2008) suggested that a change in isoform ratios could provide a flexible mechanism for adaptive responses to environmental fluctuations. The change in the proportion of isoforms with different affinities could affect the rates of specific metabolic reactions. Gillespie and Kojima (1968) were the first to discover that the degree of polymorphism depends largely on the function of the enzyme. They observed that enzymes catalysing non-specific or multiple substrates were more polymorphic than those specific for a single substrate.

Crustaceans usually show a recurring pattern of endopeptidases. For example, *Penaeus vannamei* individuals have three isoforms of trypsin. These isotrypsins are segregated according to Mendelian rules, and hence, external stimuli such as feeding do not affect the phenotype or pattern. Specimens conserve this isoform pattern throughout the digestion process (Sainz et al., 2005). In contrast, individuals of *C. crangon* show a very inconsistent isoform pattern. It varies between individuals and it is not gender-related or related to biological habits such as feeding (Teschke and Saborowski, 2005). Saborowski et al. (2012) suggested that the heterogeneous pattern of endopeptidases in *C. crangon* might reflect heterozygosity of genes coding for these enzymes. This heterogeneity of digestive enzymes may have facilitated the adaptation of *C. crangon* to an environment with variable food supply.

4.2. Lipases

Lipids are the major form of metabolic energy stores in crustaceans (Lee et al., 2006). They fuel metabolic processes, particularly during food scarcity, but also contribute significantly to cell integrity and reproduction. Lipid storage and lipid utilization are closely related to the life history traits of species (Falk-Petersen et al., 2000; Hagen and Auel, 2001). Therefore, the efficient utilization of nutritional lipids is a crucial catabolic process.

Triacylglycerol lipases are the key enzymes in lipid catabolism. They catalyse the hydrolysis of triacylglycerol (TAG), which results in the sequential liberation of fatty acids and glycerol (Watt and Steinberg, 2008). Triacylglycerol lipases are the major lipid-degrading enzymes in crustaceans (O'Connor and Gilbert, 1968). Pasquevich et al. (2011) isolated a 72 kDa lipase from the midgut gland of the prawn *Macrobrachium borellii*. The enzyme hydrolysed various TAG substrates with long-chain fatty acids. Another digestive lipase was isolated from the midgut gland of the whiteleg shrimp *Penaeus vannamei* (Rivera-Pérez et al., 2011). This lipase had a molecular mass of about 45 kDa. The full cDNA sequence showed similarities with insect lipases. The studies on decapod crustaceans cited above considered single proteins. Furthermore, a very high number of 63 lipase genes and/or gene copies were discovered in the water flea *Daphnia pulex* (Branchiopoda) and were grouped in four clusters (Koussoroplis et al., 2017). The authors studied the influence of diet and time on the expression of selected genes and identified five diet-responsive lipases including one dominant lipase. The results indicated a fine-tuned variation in the expression of lipases, reflecting a physiological adaptation to complex nutritional situations.

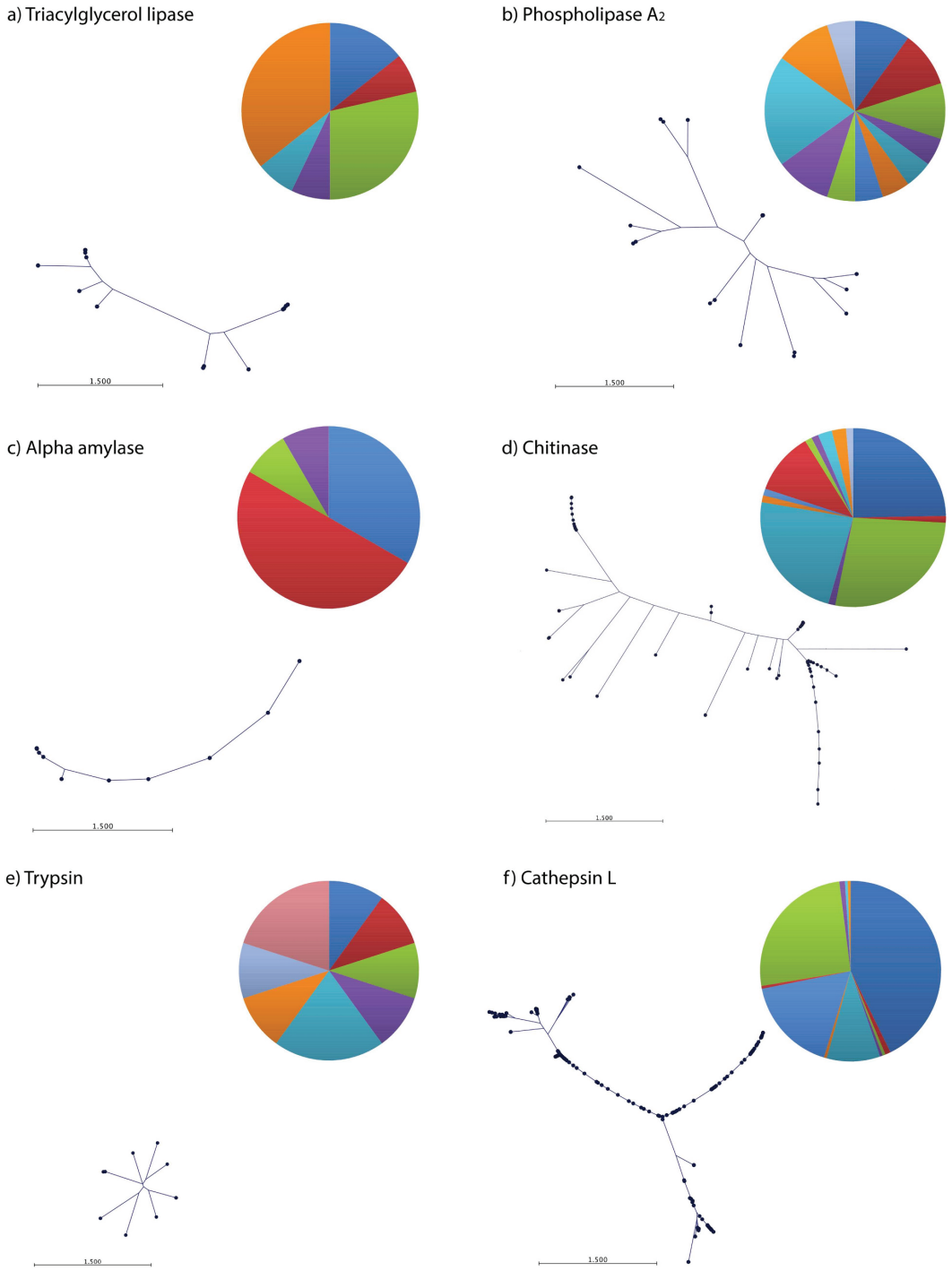

Fig. 1. Neighbor-joining cladogram of partial amino acid sequences obtained from the transcriptome of *Crangon crangon*: a) triacylglycerol lipase, b) phospholipase A_2, c) alpha amylase, d) chitinase, e) trypsin, f) cathepsin L. The pie charts represent the frequency in percentage of the putative isoforms. Every colour in the pie charts represents a different isoform.

Table 4
Top BLAST hits in NCBI with the putative enzyme isoforms of *C. crangon*.

Enzyme	EC					
	Isoform	Frequency	Top BLAST hit	Best BLASTP	*Species*	NCBI sequence ID
	ID	(%)	(Function)	Identity		
Triacylglycerol lipase (TAG)	**3.1.1.3**					
	1	29	Pancreatic TAG lipase	Pancreatic lipase-related protein 2-like (pred.)	*Hyalella azteca*	XP_018007242.1
	2	14	Pancreatic TAG lipase	Pancreatic lipase-related protein 2	*Daphnia magna*	KZS04512.1
	3	7	Pancreatic TAG lipase	Pancreatic lipase-related protein 3-like (pred.)	*Hyalella azteca*	XP_018007172.1
	4	36	Gastric TAG lipase	TAG lipase	*Portunus trituberculatus*	AHJ81100.1
	5	7	Gastric TAG lipase	Lipase 3-like	*Folsomia candida*	XP_021957766.1
	6	7	Gastric TAG lipase	Lipase 3-like (pred.)	*Nasonia vitripennis*	XP_016839047.1
Phospholipase A_2 (PL A_2)	**3.1.1.4**					
	1	5	Ca-indep. PL A_2-gamma	Ca-indep, PL A_2-gamma (pred.)	*Hyalella azteca*	XP_018021020.1
	2	5	Ca-indep. PL A_2-gamma	Ca-indep. PL A_2-gamma-like (pred.)	*Hyalella azteca*	XP_018021020.1
	3	10	PL A_2 isozymes PA3A/PA3B/PA5	Group 3 secreted PL A_2 (pred.)	*Daphnia magna*	KZS16890.1
	4	5	Group XIIA secreted PL A_2	Group XIIA secreted PL A_2	*Onthophagus taurus*	XP_022920599.1
	5	10	Group XIIB secreted PL A_2-like	PL-like A_2	*Daphnia pulex*	EFX86063.1
	6	5	Group XV PL A_2	Group XV PL A_2-like	*Zootermopsis nevadensis*	XP_021917685.1
	7	10	PL A_2, membrane associated	PL-like protein A_2	*Daphnia pulex*	EFX89898.1
	8	10	85/88 kDa Ca-indep. PL A_2	Ca-indep. PL A_2-like (pred.)	*Hyalella azteca*	XP_018010803.1
	9	10	85/88 kDa Ca-indep. PL A_2	Ca-indep. PL A_2-like (pred.)	*Hyalella azteca*	XP_018010803.1
	10	5	85/88 kDa Ca-indep. PL A_2	Ca-indep. PL A_2	*Papilio xuthus*	KPI97229.1
	11	5	Putative PL A_2, group VI	Ca-indep. PL A_2 (pred.)	*Polistes canadensis*	XP_014613252.1
	12	20	Cytosolic PL A_2	Cytosolic PL A_2	*Penaeus monodon*	AFJ11391.1
Alpha amylase	**3.2.1.1**					
	1	50	Alpha amylase	Alpha amylase	*Astacus leptodactylus*	AIW65942.1
	2	8	Alpha amylase	Alpha amylase	*Penaeus monodon*	AME17649.1
	3	33	Alpha amylase	Alpha amylase	*Panulirus argus*	CDU84835.1
	4	8	Alpha amylase	Alpha amylase	*Marsupenaeus japonicus*	AHN91843.1
Chitinase	**3.2.1.14**					
	1	28	Endochitinase	Chitinase	*Charybdis japonica*	AFF59213.1
	2	1	Endochitinase	Chitinase 1C	*Macrobrachium nipponense*	AHL24866.1
	3	1	Chitinase	Chitinase domain-containing protein (pred.)	*Hyalella azteca*	XP_018012972.1
	4	1	Chitinase	Chitinase	*Pandalopsis japonica*	AFC60662.1
	5	11	Acidic mammalian chitinase	Chitinase	*Macrobrachium nipponense*	AHL28109.1
	6	1	Acidic mammalian chitinase	Chitinase	*Pandalopsis japonica*	AFC60662.1
	7	1	Acidic mammalian chitinase	Chitinase	*Pandalopsis japonica*	AFC60662.1
	8	25	Probable chitinase 2	Chitinase	*Pandalopsis japonica*	AFC60660.1
	9	23	Probable chitinase 3	Chitinase 3 (pred.)	*Drosophila busckii*	XP_017854359.1
	10	3	Probable chitinase 3	Chitotriosidase-1	*Daphnia magna*	KZS13699.1
	11	3	Chitinase-like prot. 3	Chitinase-3-like protein 2 (pred.)	*Diuraphis noxia*	XP_015376197.1
	12	1	Chitinase-like prot. 3	Chitinase-3-like protein 2 (pred.)	*Orussus abietinus*	XP_012274144.1
	13	1	Chitinase-3-like prot. 1	Chitinase-3-like protein 2	*Nilaparvata lugens*	XP_022196567.1
Trypsin	**3.4.21.4**					
	1	20	Trypsin	Trypsin, partial	*Litopenaeus vannamei*	CAA75311.1
	2	20	Trypsin	Trypsin	*Macrobrachium rosenbergii*	AMQ98968.1
	3	10	Trypsin	Trypsin, partial	*Litopenaeus vannamei*	CAA75311.1
	4	10	Trypsin	Trypsin 1a	*Panulirus argus*	ADB66711.1
	5	10	Trypsin	Trypsin 3	*Panulirus argus*	ADB66714.1
	6	10	Trypsin	Trypsin	*Euphausia superba*	AOW41609.1
	7	10	Trypsin	Trypsin	*Euphausia superba*	AOW41609.1
	8	10	Cationic trypsin	Trypsin	*Euphausia superba*	AOW41605.1

(*continued on next page*)

Table 4 (*continued*)

Enzyme	EC					
	Isoform	Frequency	Top BLAST hit	Best BLASTP	*Species*	NCBI sequence ID
	ID	(%)	(Function)	Identity		
Cathepsin L	**3.4.22.15**					
	1	1	Cathepsin L	Cathepsin L	*Riptortus pedestris*	BAN20648.1
	2	1	Cathepsin L	ND		
	3	7	Cathepsin L1	Cathepsin L1-like (pred.)	*Monodelphis domestica*	XP_001367224.2
	4	21	Cathepsin L2	Cathepsin L	*Palaemon carinicauda*	AGJ03550.1
	5	1	Cathepsin L2	Cathepsin L	*Palaemon carinicauda*	AGJ03550.1
	6	1	Cathepsin L2	Cathepsin L	*Palaemon carinicauda*	AGJ03550.1
	7	1	Cathepsin L2	Cathepsin L	*Marsupenaeus japonicas*	AJS11553.1
	8	1	Cathepsin L2	Cathepsin L	*Palaemon carinicauda*	AGJ03550.1
	9	17	Crustapain	Cathepsin L	*Palaemon carinicauda*	AGJ03550.1
	10	10	Crustapain	Cathepsin L	*Palaemon carinicauda*	AGJ03550.1
	11	1	Crustapain	Cathepsin L, partial	*Palaemon varians*	ACR54126.1
	12	1	Crustapain	Cathepsin L, partial	*Palaemon varians*	ACR54126.1

Putative isoforms of the studied enzymes and their frequency of appearance. Top BLAST hits after annotation of the transcriptome of *C. crangon* and the best similarity found in the database of the National Center for Biotechnology Information (NCBI). Pred. = Predicted, Ca-ind. = Calcium independent.

Reports about lipases in decapod crustaceans are scarce and to date information about putative isoforms is lacking. In the present work, however, we identified 14 transcripts of triacylglycerol lipase in the hepatopancreas of *C. crangon*. The transcripts apparently belong to six putative isoforms (Table 3). The major part of the transcripts corresponds to two main isoforms (Fig. 1a). These dominant isoforms show highest similarity with the pancreatic triacylglycerol lipase and the gastric triacylglycerol lipase of mammals, but also with crustacean and insect lipases (Table 4). These results indicate that *C. crangon* may have at least two triacylglycerol lipase isoforms. This suggestion is supported by electrophoretic results of the hepatopancreas of *C. crangon*, which show two main activity bands, a heavy one of about 150 kDa and a light one of about 57 kDa, and several minor activity bands (Saborowski, pers. comm.)

Different to TAG lipases, the phospholipase A_2 superfamily is a group of lipolytic enzymes that hydrolyse the sn-2 position in phospholipids to release free fatty acids (reviewed by Dennis et al., 2011). Basically, these phospholipases are separated into cytosolic enzymes and those, which are secreted (Leslie, 2015; Yamamoto et al., 2017). The various catalytically active members of the phospholipase A_2 family are classified as *e.g.* secreted phospholipase A_2 ($sPLA_2$), cytosolic phospholipase A_2 ($cPLA_2$), calcium-independent phospholipase A_2 ($iPLA_2$), or lysosomal phospholipase A_2 ($LPLA_2$) (Dennis et al., 2011).

Within this study, we found 20 transcripts of phospholipases A_2 with 12 putative isoforms (Table 3). According to their sequences, most of the putative isoforms fit into the above mentioned classification of phospholipases A_2 (Table 4). The most recurrent isoform was the calcium-independent phospholipase A_2 ($iPLA_2$), which does not require Ca^{2+} for activity, is ubiquitously expressed and participates in lipid catabolism (Barbour and Ramanadham, 2017). This isoform may also play a major role in membrane phospholipid remodelling (Murakami and Kudo, 2002). Overall, the high number of isoforms of phospholipase A_2 in the *C. crangon* transcriptome seems to reflect the various processes, in which this enzyme is involved. Enzymes involved in metabolic processes are expected to have a low level of polymorphism. However, each of the isoforms in the mentioned classification of phospholipase A_2 has a different function. Therefore, high polymorphism is expected.

4.3. Carbohydrases

Among carbohydrases, alpha amylase is one of the dominant enzymes in the midgut gland of crustaceans and other invertebrates (Van Wormhoudt et al., 1995; Pavasovic et al., 2006; Fuzita et al., 2015; Rodríguez-Viera et al., 2016). Alpha amylase hydrolyses alpha-1,4 glycoside bonds in starch and glycogen. This enzyme proved to be a suitable model for investigations of adaptive evolutionary processes of species with different feeding habits (Rodríguez-Viera et al., 2016). Van Wormhoudt et al. (1995) studied the polymorphism of alpha amylase in 40 different species of decapods. The authors found that omnivorous crustaceans have a higher number of isoforms (5 to 6) than carnivorous crustaceans (1 or 2). In *C. crangon* we identified four putative isoforms (Table 3), most transcripts correspond to two dominant isoforms (Fig. 1c). These results reflect the omnivorous and opportunistic feeding behaviour of *C. crangon*. In comparison with the other enzymes analysed in this study, alpha amylase shows the lowest number of putative isoforms.

It is possible that after post-translational modifications the number of isoforms increases. Rodriguez-Viera et al. (2016) studied polymorphism of alpha amylase in the spiny lobster *Panulirus argus*. The authors found that the two isoforms of alpha amylase in the lobster are the result of post-translational modifications of the same gene that encodes a conserved alpha amylase protein. The most frequent alpha amylase phenotype showed the lowest digestive efficiency. In the spiny lobster the gradual digestion of carbohydrates and liberation of glucose to the hemolymph accelerates the post-absorptive utilization (Rodríguez-Viera et al., 2014). The predominance of the isoform with low digestion efficiency seems to be a favoured evolutionary trait to control excessive carbohydrate digestion of the lobster (Rodríguez-Viera et al., 2016).

Chitinase randomly hydrolyses the *endo*-β(1 → 4) linkages between the *N*-acetyl-β-D-glucosaminide monomers in chitin. In crustaceans, chitinases have several functions associated with various processes such as moulting, disease resistance, and digestion of chitinous food. Moreover, the expression of chitinases depends on the tissue. The Antarctic krill, *Euphausia superba,* for example, expresses at least two distinct forms of chitin-degrading enzymes, which, again, show several isoenzymes involved either in moulting or in digestion (Peters et al., 1999; Saborowski and Buchholz, 1999). At least four major groups of chitinases were described in crustaceans (Rocha et al., 2012; Salma et al., 2012; Zhang et al., 2014; Li et al., 2015; Zhou et al., 2017). Chitinases of group 1 facilitate the digestion of chitin-containing food (Rocha et al., 2012; Salma et al., 2012; Zhang et al., 2014; Li et al., 2015). Chitinases of group 2 are involved in the degradation of the exoskeleton (Rocha et al., 2012; Salma et al., 2012; Li et al., 2015). Chitinases of group 3 have a dual role: digestion of chitin food and defence against pathogens (Salma et al., 2012; Li et al., 2015). Chitinases of group 4 are the least studied, but they may play a role in immune defences against pathogen infection (Zhou et al., 2017).

In the transcriptome of *C. crangon* we detected 80 transcripts of

chitinase and 13 putative isoforms (Table 3). Cladogram analysis and pie charts (Fig. 1d) showed at least four main isoforms belonging to different chitinase groups. We mainly found transcripts from group 3, followed by transcripts from group 2. Cladogram analysis shows sequence similarity of isoforms according to the group they belong to. The high level of polymorphism in chitinase transcripts of *C. crangon* seems to be related to the different functions of chitinases in crustaceans. The highest number of transcripts was associated with food digestion, which emphasizes the midgut gland as the major digestive organ.

4.4. Proteases

In crustaceans the hydrolysis of food protein is a crucial step in digestion (Muhlia-Almazán et al., 2008). Most crustacean species use the serine protease trypsin for the degradation of alimentary protein (Klein et al., 1996; Celis-Guerrero et al., 2004; Muhlia-Almazán et al., 2008; Navarrete-Del-Toro et al., 2011; Navarrete-Del-Toro et al., 2015). In crustaceans the number of trypsin isoforms is variable. For example, *Penaeus vannamei* has three isoforms (Sainz et al., 2005) and the lobster *Panulirus argus* has at least two isoforms (Perera et al., 2012). In *C. crangon,* however, previous reports about activity bands after separation by electrophoresis suggested high polymorphism of trypsin in the midgut gland (Saborowski et al., 2012). Our results support this suggestion, as they revealed ten different transcripts (Table 3). These transcripts are not related to a preferential isoform (Fig. 1e). Such a high polymorphism of trypsin in *C. crangon* may indicate a strategy to efficiently utilize alimentary protein from various sources.

The cysteine endopeptidase cathepsin L is also involved in protein degradation. In crustaceans this enzyme appears to be less important for the digestion of dietary protein compared to the serine proteinases trypsin and chymotrypsin. *C. crangon* seems to be an exception, because its activity of cysteine proteinases is high and, thus, appears to play an important role in food digestion (Teschke and Saborowski, 2005). According to our results the number of transcripts of the cysteine proteinase cathepsin L is higher than that of the serine proteinase trypsin or any other enzyme analysed here (Table 3). The information presented here also shows that cathepsin L has a quite low degree of polymorphism in the brown shrimp *C. crangon.* Cladogram analysis and the pie chart indicate that almost half of the transcripts represent the same isoform (Fig. 1f).

5. Conclusion

Utilizing transcriptome data of midgut glands, we showed that trypsin and other digestive enzymes exhibit high levels of polymorphism in the brown shrimp *C. crangon*. This polymorphism varies among the different enzymes: in lipases and carbohydrases a higher number of isoforms was found in enzymes that - besides their extracellular functions - also have diverse intracellular functions. We suggest that polymorphism of digestive enzymes in *C. crangon* represents an adaptive strategy, as the variability of food quality and quantity changes drastically between seasons in the North Sea. Enzyme polymorphism may provide *C. crangon* with the ability to utilize a wide spectrum of substrates as energy sources.

Data accessibility

The sequence data have been submitted to the GenBank databases under the following accession numbers: triacylglycerol lipases (MH055763-MH055776), phospholipase A_2 (MH055777-MH055796), alpha amylase (MH055751-MH055762), chitinase (MH069216-MH069295), trypsin (MH035874-MH035882, MH069215), cathepsin L (MH069296-MH069494). The BioProject ID of our data is PRJNA479562, and the BioSample accession number is SAMN09580251. All raw reads were deposited into the Sequencing Read Archive (SRA) of NCBI with accession number SRP152406. This Transcriptome Shotgun Assembly project has been deposited at DDBJ/ENA/GenBank under the accession GGRU00000000. The version described in this paper is the first version, GGRU01000000.

Author contributions

D.M.A., WH and R.S designed the research. D.M.A and R.S performed the research. D.M.A. and L.H contributed bioanalytical tools. D.M.A, L.H., and R.S. analysed the data. D.M.A and R.S. wrote the manuscript. D.M.A., L.H., W.H. and R.S. contributed to the critical appraisal of the paper and approved the final version.

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3.4 Publication III

Molecular aspects of lipid metabolism in the midgut gland of the brown shrimp *Crangon crangon.*

Diana Martínez-Alarcón, Wilhelm Hagen, Christoph Held, Reinhard Saborowski

to be submitted to

Comparative Biochemistry and Physiology Part B: Biochemistry & Molecular Biology

Molecular aspects of lipid metabolism in the midgut gland of the brown shrimp *Crangon crangon*

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ABSTRACT

The shrimp *Crangon crangon* is well adapted to the variable environmental conditions in the southern North Sea. It is very abundant, has high reproduction rates and holds a key position in coastal ecosystems. Surprisingly, this species has very low lipid deposits in the midgut gland, suggesting that the midgut gland functions as a metabolic center rather than a storage organ. Based on seasonal gene expression studies and established transcriptome data, we investigated key components of lipid metabolic pathways. Gene expression of triacylglycerol lipase, phospholipase and fatty acid desaturase were analyzed and compared with those of other digestive enzymes involved in lipid, carbohydrate and protein catabolism. Our results suggest that gene expression of digestive enzymes involved in lipid metabolism is modulated by the lipid content in the midgut gland and is related to food availability. Brown shrimps seem to be capable of using cellular phospholipids during periods of food paucity but high energetic (lipid) requirements. Three of four isoforms of fatty acid binding proteins (FABPs) from the midgut gland involved in fatty acid transport showed specific disadvantageous mutations of the binding site. Additionally, Enzymes involved in the formation of storage lipids (triacylglycerols) showed quite low numbers of transcripts. We hypothesize that the mutations in FABP, and deficiencies in anabolic pathways limit lipid storage capacities in the midgut gland of *C. crangon*. In turn, food utilization, including lipid catabolism, has to be efficient to fulfill the energetic requirements of brown shrimps.

1 Introduction

The brown shrimp *Crangon crangon* is a key species of the southern North Sea ecosystem with major commercial importance. It has high reproduction rates and is well adapted to the variable environmental conditions. Lipid stores are important energy reserves to survive periods of food scarcity, especially in species confronted with highly variable conditions. However, *C. crangon* shows rather low lipid levels of about 14-17% of dry mass (%DM) in the midgut gland, the typical storage organ in decapods. In comparison, the midgut gland of the pink shrimp *Pandalus montagui*, which also inhabits southern but also central parts of the North Sea, has lipid levels of 47-70%DM (Martínez-Alarcón et al. 2019b). Therefore, it has been suggested that the midgut gland of *C. crangon* functions as a metabolic center rather than a storage organ (Clarke 1982, Martínez-Alarcón et al. 2019b).

In invertebrates, lipid metabolism is highly coordinated and involves multiple catabolic and anabolic processes. The lipid metabolism of crustaceans has been poorly investigated. Studies of their catabolic capacities showed dynamic gene expression of two lipases after starvation of the shrimp *Penaeus vannamei* (Rivera-Pérez & García-Carreño 2011). In the crayfish *Cherax quadricarinatus* a lipase was differentially expressed during moulting and fasting periods (Yudkovski et al. 2007). At the transcriptional level, dietary lipids had obvious effects on fatty acid biosynthesis and ß-oxidation in the hepatopancreas of the Chinese mitten crab *Eriocheir sinensis* (Wei et al. 2017). The transcriptome of *C. crangon* exhibits a high level of polymorphism in triacylglycerol lipase and phospholipase A_2 (Martínez-Alarcón et al. 2019a) indicating a high flexibility of the lipid catabolism.

In addition to the catabolic digestive lipases, other proteins without catalytic power also have key functions in the lipid metabolism, for example those involved in lipid transport. The transfer of lipids between organs and organelles is an essential biological process. Inside and outside the cell, specialized proteins redistribute the hydrophobic lipid molecules (Van der Horst & Ryan 2012). There is a large amount of different lipid transfer proteins that have been described so far. The metabolic functions of those molecules are targeted by several investigations. However, the majority of these studies focus on the lipid metabolism of insects (Van der Horst & Ryan 2012) or humans (Chen & Davidson 2012). There are still many gaps in our knowledge of this topic, especially concerning crustacean lipid metabolism. Furthermore, the vast majority of research done on lipid transport of crustaceans is mostly focused on the transport between different tissues through the hemolymph (O´Connor & Gilbert 1968), rather

than the transport of lipid molecules within cells. There are vesicular and non-vesicular routes for intracellular traffic. In non-vesicular routes, the so-called lipid transfer proteins (LTPs) mediate lipid transfer across the cytoplasm. LTPs stimulate the extraction of lipids from a membrane, their mobilization in the cytoplasm and their re-insertion into different membranes (Wong et al. 2017). LTP specificity varies, but many of them shield the hydrophobic portion of lipids in internal cavities. In this way lipids are transported in a mobile protein segment into the cytoplasm. There are several families of proteins that are capable of mobilize lipids (Wong et al. 2017). Also, in the cytoplasm the flux of fatty acids between organelles seems to be controlled by fatty acid-binding proteins (FABPs) (Maatman et al. 1994). These proteins bind non-covalently to fatty acids and were identified for instance in the Chinese mitten crab (Gong et al. 2010). A thorough review of invertebrate intracellular FABPs is given by Esteves & Ehrlich (2006).

Metabolic pathways for the synthesis of triacylglycerols (TAG) and phospholipids (PL) were already proposed by Kennedy & Weiss (1956), in which several enzymes are involved. The Kennedy pathway is the principal route to TAG synthesis. This pathway involves the addition of fatty-acyl groups to a glycerol-3-phosphate to form phosphatidic acid (PA), which is converted to diacylglycerol (DAG) by phosphatidic acid phosphatase; DAG is further acylated by diacylglycerol acyltransferase and forms TAG (Kennedy & Weiss 1956).

The aim of this study is to investigate principal components of the lipid metabolism in the midgut gland of the brown shrimp *Crangon crangon* to better understand its function and, particularly, its limited lipid storage capacities. Therefore, we addressed the following objectives: 1) to study seasonal expression patterns of key lipase digestive enzymes, 2) to compare the gene expression of lipases with that of other important digestive enzymes, 3) to analyze the occurrence and structure of FABPs, and 4) to identify enzymes of principal anabolic pathways.

2 Material and methods

2.1 Origin of samples

Brown shrimps *Crangon crangon* (Linnaeus, 1758) were collected by bottom trawling with R/V *Uthörn* during four sampling campaigns. Sampling was carried out in February, April, July and October 2016 in the southern North Sea near the island of Helgoland (Table 1). Total body length (rostrum to tip of telson) and sex of adult animals were determined onboard the ship.

Thereafter, animals were dissected and midgut glands (30 – 50 mg) were transferred to individual 1.5 ml reaction tubes containing 0.5 ml of RNAlater (AM7020, Life Technologies, Ontario, Canada). The samples were shock-frozen in -80°C, shipped frozen to the laboratories of the Alfred Wegener Institute in Bremerhaven and kept at -80°C until further processing and analysis.

2.2 Reference genes and primer design

Forward and reverse primer sequences used for the qPCR are shown in Table 2. The cell skeleton protein ß actin, the chromatin protein histone H3 and the hypoxia-inducible factor (HIF) were selected as possible reference genes for the normalization of target gene expression. The choice of these reference genes was based on a similar analysis by Koenigstein et al. (2013). We focused our gene expression study on selected enzymes involved in lipid metabolism (fatty acid desaturase, phospholipase, triacylglycerol lipase), carbohydrate digestion (alpha-amylase, chitinase) and protein utilization (trypsin and cathepsin L). All primers in the study were designed based on the sequence information obtained from the transcriptome data (Martínez-Alarcón et al. 2019a), using Primer-Blast from NCBI (National Center for Biotechnology Information). Candidate primer pairs were double-checked for primer dimers using Oligo Analyzer 3.1. Each primer pair was evaluated on the grounds of the absence of artifacts in their melting curve and their efficiency was assessed by serial template dilution. The specificity of the RT-qPCR amplification was confirmed by melt analysis of the reactions and by sequencing of the PCR products.

2.3 Extraction of mRNA from midgut glands

Midgut gland samples of *C. crangon*, previously stored in RNAlater at -80°C, were thawed and about 30 mg of the tissue was used for RNA extraction. The Precellys Keramik Kit (PEQLAB 91-PCS-CKM, Erlangen, Germany) was used for cell lysis by three cycles of 15 s shaking and 30 s pauses in between shakes. Thereafter, samples were centrifuged at 13,000 g for 3 min at room temperature. Total RNA was isolated using RNeasy Mini Kit spin columns (Qiagen 74104, Hilden, Germany) following the manufacturer´s instructions. RNA quantities and purities were determined with a NanoDrop ND-1000 device at 260 nm and 260/280 nm, respectively. A high capacity cDNA RT Kit (Applied Biosystems, USA) was used to generate cDNA.

2.4 Real-time qPCR

Gene expression levels were studied by real-time PCR (q-PCR, Rotor-Gene Q, Qiagen). The tested samples were 100-fold diluted and the primers were used in a final concentration of 700 nM in a Master Mix (HRM kit Qiagen) in 0.1 ml Qiagen tubes for the Rotor-Gene. Every sample was tested in duplicate. The PCR amplification programme was 5 min at 95°C, followed by 40 cycles of 10 s at 95°C, and 30 s at 55°C. PCR products were sequenced and the results were analyzed in CodonCodeAligner software and compared for matching with the sequences from the NCBI database.

2.5 Data Processing and statistics

Relative levels of gene expression were calculated by the Comparative Quantification feature in the Rotor-Gene Q Series software V. 1.7.94 (Qiagen) with the deltadeltaC_T method (Joehanes & Nelson 2008). Data were transferred to Excel (Microsoft) and the stability of the candidate reference genes was tested with the macros GeNorm (Vandesompele et al. 2002) and NormFinder (Andersen et al. 2004). RStudio software, version 0.99.491 was used to prepare the graphs.

2.6 Fatty Acid Binding Protein (FABPs) sequences and 3D structure

Sequences from the transcriptome of the midgut gland of *C. crangon* (Martínez-Alarcón et al. 2019a) identified as FABPs were aligned using ClustalW at EMBL-EBI (Larkin et al. 2007) against ten other previously reported FABPs in NCBI database and two sequences reported by Söderhäll et al. (2006) (Table 3). A cladogram based on protein sequences was constructed using CLC Genomics Workbench version 12.0.1 (QIAGEN). Evolutionary history was interfered by the Maximum Likelihood method based on the Jukes-Cantor model (Jukes & Cantor 1969). The initial tree was obtained by applying the Neighbor Joining method. A bootstrap analysis with 10,000 replicates was performed for assessing confidence in the analysis of the clades. 3D structures of the different isoforms of *C. crangon* FABPs were predicted with the online software SWISS-MODEL (Waterhouse et al. 2018).

2.7 Transcriptome screening for enzymes and proteins involved in lipid metabolism

Transcriptome data of *C. crangon* midgut glands, previously reported by Martínez-Alarcón et al. (2019a), was screened for the presence of enzymes involved in the catabolism and

anabolism of alimentary lipids. Particular attention was paid to the enzymes involved in the Kennedy pathway facilitating the synthesis of triacylglycerols (TAG) and diacylglycerols (DAG).

3 Results

3.1 Identification of reference genes

Candidate reference gene expression stability was calculated by two algorithms (GeNorm and NormFinder). Both algorithms identified actin as the most stable gene, hypoxia-inducible factor (HIF) ranked second, and histone 3 (H3) was the least stable gene (Table 4). GeNorm calculates an average expression stability M based on the standard deviation between all genes and samples, while NormFinder returns a model-based stability value between groups (Koenigstein et al. 2013).

3.2 Seasonal expression of digestive enzyme genes

The digestive enzyme triacylglycerol lipase involved in the catabolism of TAG showed deviating expressions between seasons. From autumn to winter this enzyme was down-regulated ($p= 0.008$), and up-regulated from winter to summer ($p= 0.004$) (Fig. 1a). The gene expression of phospholipase, which is involved in the catabolism of phospholipids (Fig. 1b) and fatty acid desaturase, important for the anabolism of lipids, did not show statistical differences between seasons (Fig. 1c). The ratio of gene expression between phospholipase and triacylglycerol lipase showed significant seasonal changes (Fig. 2). In spring, expression of phospholipase was double as high as the expression of triacylglycerol lipase. In summer, the ratio changed and the expression of triacylglycerol lipase became higher. This tendency continued in autumn, showing double as high expression of triacylglycerol lipase than phospholipase. Towards winter, the ratio changed again, showing a five times higher expression of phospholipase compared to triacylglycerol lipase.

3.3 Correlation analysis of digestive enzyme gene expression

Seasonal gene expression of phospholipase was always negatively correlated with the other digestive enzymes. The correlation coefficient with triacylglycerol lipase was -0.17 ($p = 0.31$) and with fatty acid desaturase -0.29 ($p = 0.09$). Also, with cathepsin L, involved in protein catabolism, phospholipase showed a negative correlation coefficient of -0.13 ($p = 0.47$). Comparison with carbohydrases produced correlation coefficients of -0.31 ($p = 0.07$) for

chitinase and -0.20 ($p = 0.24$) for alpha amylase. Strong positive relationships were determined for triacylglycerol lipase and cathepsin L 0.52 ($p < 0.001$), for triacylglycerol lipase and chitinase 0.53 ($p< 0.001$), and for trypsin and cathepsin L 0.64 ($p < 0.001$) (Table 5).

3.4 Alignment and cladogram of fatty acid binding protein (FABP) sequences

BLAST analysis confirmed the coding sequences obtained from the transcriptome of the midgut gland of *C. crangon* (Martínez-Alarcón et al. 2019a) as FABPs. The four isoforms of FABPs were aligned with other previously reported FABPs in the NCBI database (Table 3). Secondary structure characteristics of FABP proteins were identified in the four isoforms of *C. crangon*. One of the three amino-acid residues of the P2 motif, which is characteristic for FABPs (Söderhäll et al. 2006) (involved in the interaction with ligand carboxylate) was found mutated in two isoforms of *C. crangon* FABPs. This mutation substituted valine (V) in the position of arginine (R) 110. This substitution was found in the isoforms 3 and 4 of *C. crangon* but not in the isoforms 1 and 2. The isoform 2 contained another mutation in the P2 motif, where tyrosine (Y) was substituted by a phenylalanine (F) 132 (numbers according to *P. monodon* sequence) (Fig. 3).

A cladogram was created with the FABP sequences from the midgut gland of *C. crangon* and several other FABPs reported in the NCBI data base (Table 3). We obtained two main branches, one with *Homo sapiens* sequences and the other with crustacean sequences. Two separate branches represented the insect *Drosophila melanogaster* and the trematode *Schistosoma japonicum*. Within the crustacean branch, the cladogram showed that the isoforms 2 to 4 of *C. crango*n are closely related. Isoform 1 of *C. crangon* is more closely related to *Pacifastacus leniusculus* than to the other three isoforms of *C. crangon* (Fig. 4).

3.5 3D structure of C. crangon FABPs

3D structures of the four isoforms of FABP found in the midgut gland of *C. crangon* are shown in Fig. 5. Isoform 1 and 2 possess the three binding residues characteristic of the FABP family (Arg110, Arg130, Tyr132) (number based on *P. monodon* sequence). Isoforms 3 and 4 have a valine (V) residue instead of arginine (R). The four isoforms present the α helix and β strands characteristic of FABPs.

3.6 Enzymes and proteins involved in lipid metabolism

Proteins involved in lipid metabolism found in the transcriptome of the midgut gland of *C. crangon* (Martínez-Alarcón et al. 2019a) are listed in Table 6. We focused on key proteins involved in lipid catabolism, anabolism, and transport.

Lipophorin (LPP), which transports lipids in the intracellular space to other organs, showed the highest number of transcripts, followed by glycerol-3-phosphate acyltransferase (GPAT), which participates in the synthesis of triacylglycerols, and phospholipase A_2 (PLA_2) that is involved in the catabolism of phospholipids. But we detected low numbers of transcripts of other enzymes that are involved in the last steps of triacylglycerol synthesis: two transcripts of phosphatidic acid phosphohydrolase (PAP) and only one transcript of the acylglycerophosphate acyltransferase (AGPAT). Finally, we found nine transcripts of diacylglycerol acyltransferase (DGAT), a key enzyme involved in the last step of TAG synthesis (Table 6).

4 Discussion

4.1 Seasonal gene expression of digestive lipases

Triacylglycerol (TAG) lipases hydrolyze dietary triacylglycerol (Watt & Steinberg 2008). The expression of TAG lipase in the midgut gland of *C. crangon* followed a distinct seasonal cycle. The up-regulation of this enzyme from spring over summer to autumn parallels the seasonal cycle of primary and secondary production. Phytoplankton production in the southern North Sea starts with a spring bloom between March and June and continues at a lower level until autumn (Wiltshire et al. 2015). Hence, the expression of TAG lipases apparently reflects the utilization of dietary TAG during the productive period. Concomitantly, total lipid levels in the midgut glands increased towards autumn (Martínez-Alarcón et al. 2019b), supporting this assumption. Moreover, expression of TAG lipase was positively correlated with the expression of chitinase, especially during spring ($r^2 = 0.66$) and summer ($r^2 = 0.81$), and with the proteinase cathepsin L ($r^2 = 0.52$), indicating that *C. crangon* is capable of utilizing chitin and protein from prey organisms such as smaller crustaceans and polychaetes (Plagmann 1939, Pihl & Rosenberg 1984). The high correlation of the gene expression between the two analyzed proteases (trypsin and cathepsin L) suggests that both enzymes are co-expressed.

Gene expression of phospholipase A_2, in contrast, did not show a clear seasonal pattern and it did not correlate with productivity, since expression was higher in winter than in other seasons. Phospholipases A_2 form a superfamily of enzymes that release free fatty acids by

hydrolysis of phospholipids at the sn-2 position (reviewed by Dennis et al. 2011). Phospholipids are the main components of biomembranes and, therefore, important in maintaining cell integrity. Phospholipases contribute to the intracellular and extracellular hydrolysis of phospholipids (Leslie 2015; Yamamoto et al. 2017). Our results showed a slight up-regulation of phospholipase gene expression during winter. This increase is, however, unlikely related to more intense feeding activities, since neither lipid content nor trophic marker indices increased during winter (Martínez-Alarcón et al. 2019b). Likewise, no up-regulation of gene expression of other digestive enzymes was observed.

The midgut gland is considered as the major metabolic and storage organ in crustaceans (Vogt 2019). It accounts on average for about 5% of body mass, but may even reach 8-10% in well-fed and lipid-rich species, or decrease to about 2% in starving species. Accordingly, this parameter, usually expressed as midgut gland index or hepatosomatic index (HSI= $m_{HP} \cdot 100 / m_{total}$) (m=mass), is often used as an indicator of the nutritional status of an animal (e.g. Chu 1999, Sánchez-Paz et al. 2007). Previous studies on the feeding ecology and physiology of *C. crangon* showed that the HSI of freshly caught specimens from summer and autumn ranged between 4 and 5. Acute starvation for ten days caused a rapid decrease of the HSI to half of the initial values. Simultaneously, the total lipid content decreased from 15% to 5%DM (Pöhlmann, pers. comm.). Accordingly, upon starvation these shrimps utilize a significant fraction of their midgut gland, which contains only minor lipid reserves in the form of TAG, but plenty of polar lipids as components of biomembranes (Martínez-Alarcón et al. 2019b).

Phospholipase A_2 enzymes may in fact contribute to the metabolic utilization of phospholipids in the midgut gland during periods of food scarcity. The transcriptome of *C. crangon* shows at least twelve putative isoforms of phospholipase A_2 (Martínez-Alarcón et al. 2019a). The most frequent isoform is the calcium-independent phospholipase A_2, which may play a major role in membrane phospholipid remodeling (Murakami & Kudo 2002). This suggestion is supported by the seasonal course of the gene expression ratios between triacylglycerol lipases and phospholipases (Fig. 2). During the productive seasons the relative expression of TAG lipases is higher than that of phospholipases, indicating the primary utilization of dietary triacylglycerols. In early spring and particularly in winter, when food is scarce, expression of phospholipases seems to favour utilization of intracellular phospholipids. Between November and April up to 75% of brown shrimps showed signs of starvation (Hufnagl et al. 2010). The degradation of midgut gland biomembrane is unlikely as rapid and drastic in

nature as reported above from the acute starvation experiment. The shrimps will not entirely run out of food and the low winter temperatures will strongly reduce metabolic rates and, thus, the steady energy demand. A minimum level of polar lipids (ca. 2-3%DM) is essential to maintain the physiological function of the biomembranes, cells and the organ. Besides the midgut gland, other tissues like the large abdominal muscle assemblage may provide phospholipids for energetic purposes. However, the utilization of phospholipids appears to be an emergency strategy, after most TAG has been depleted. This view agrees with a recently proposed concept considering plasma membranes as capacitors for energy and metabolism (Ray et al. 2016). Membrane compounds may act as storage molecules that can be utilized under stress conditions, providing energy, signaling molecules, and various metabolites. So far, only polar euphausiids, e.g. Antarctic krill *Euphausia superba*, are known to accumulate significant amounts of phospholipids (in the form of phosphatidylcholine) as energy reserves to buffer the pronounced seasonal food supply in polar oceans (Hagen et al. 1996, 2001).

Fatty acid desaturases (FAD) are involved in the anabolism of lipids. They introduce double bonds into acyl chains, thus generating unsaturated fatty acids (Los & Murata 1998). We found a positive correlation between the expression of FAD and TAG lipases, especially during autumn and winter, but a negative correlation with the expression of phospholipase A_2. These results suggest that in autumn with still sufficient food available, the midgut gland of *C. crangon* up-regulated the expression of triacylglycerol lipase to hydrolyze TAG from the food, and simultaneously, up-regulated the fatty acid desaturase. This enzyme is essential for the modulation and proper functioning of biomembranes, which are very rich in 20:5(n-3) and 22:6(n-3) polyunsaturated fatty acids. Fatty acid desaturases provide the membranes with the necessary fluidity by introducing double bonds into the fatty acids (Los & Murata 1998). In winter, when food becomes scarce, the midgut gland down-regulates triacylglycerol lipase and fatty acid desaturase and, instead, up-regulates the phospholipase A_2, which supports the utilization of fatty acids from biomembrane-derived phospholipids.

4.2 Cellular transport of lipids

The cytosolic fatty acid binding proteins (FABPs) bind non-covalently to hydrophobic ligands, mainly fatty acids, and facilitate their intracellular transport (Esteves & Ehrlich 2006). Although FABPs show variable sequences, all of them have a conserved tertiary structure. This comprises ten anti-parallel ß-strands forming a ß-barrel and helix-turn-helix motifs, delimiting the binding

cavity for the ligands (Esteves & Ehrlich 2006). A triad of amino acids that are involved in the binding with the ligand remains, however, highly conserved.

Few FABPs from invertebrates have been reported so far, showing low sequence similarities among each other (Esteves & Ehrlich 2006). Information about crustacean FABPs is even scarcer. In the crayfish *Pacifastacus leniusculus* and the shrimp *Penaeus monodon* two FABPs from hemocytes were isolated and characterized. Both proteins show the binding triad residues involved in the interaction with the ligand (Söderhäll et al. 2006). We found at least four main FABP isoforms in *C. crangon*. All of these four *C. crangon* FABPs possess the characteristic ten anti-parallel ß-strands forming a ß-barrel and two α helices. Surprisingly, three of these four isoforms in *C. crangon* show a substitution in one of the amino acid residues of the P2 motif characteristic of RA-binding proteins and FABPs (Söderhäll et al. 2006). This amino acid, in concert with two others, is important for the proper function of the FABPs, because they are involved in the interaction with the ligand. Since three of the four isoforms show mutations in one of those amino acids, the interaction with the ligands, i.e. fatty acids, may be hampered and, thus, lipid metabolism impaired.

4.3 Lipid anabolism and TAG storage

Alimentary lipids are digested by gastric TAG lipases, phospholipases, other unspecific digestive lipases, and esterases (Saborowski 2015). The resulting fatty acids and glycerides interact with membrane proteins, which facilitate their vesicular or non-vesicular transfer into the cell. Once inside the cell, the molecules can follow different pathways, depending on the physiological requirements of the organism. Metabolic energy is gained from fatty acids via β-oxidation. Other products, such as glycerol, phosphate, choline or ethanolamine contribute to the cellular pool of metabolites. The anabolic formation of TAGs as storage lipids follows the Kennedy pathway (Kennedy & Weiss 1956), which is the most important route to TAG synthesis.

In this pathway, the enzymes glycerol-3-phosphate acyltransferase (GPAT), acylglycerophosphate acyltransferase (AGPAT), phosphatidic acid phosphohydrolase (PAP) and diacylglycerol acyltransferase (DGAT) successively facilitate the synthesis of diacylglycerols (DAGs) and TAGs. The DAGs may be modified via the Lands cycle (Lands 1958) and incorporated into cell membranes. The TAGs may be transported with the help of FABPs or LTPs and stored as lipid droplets. On demand, these lipid depots can be catabolized by phospholipases and TAG lipases, respectively.

The transcriptome data of *C. crangon* includes 22 transcripts of GPAT, the enzyme catalysing the first step of the Kennedy pathway forming a monoacylglycerol. AGPAT, the enzyme for the second step, which is the formation of phosphatidic acid, a key intermediate in the biosynthesis of glycerolipids, is present with only one transcript. The enzyme for the next step (PAP), which participated in the dephosphorylation of phosphatidic acid and formation of a diacylglycerol, appears with only two transcripts. DGAT is the key enzyme in the biosynthesis of TAGs (Yen et al. 2008; Turchetto-Zolet et al. 2011). No alternative pathways are known.

The transcriptome obtained by Martínez-Alarcón et al. (2019a) contains nine transcripts of DGAT. The alignment of the deduced amino acid sequences shows low similarity (<50%) with primates and chelicerates and slightly higher similarity (66%) with the shrimp *Penaeus vannamei.* However, the obtained sequences are short and probably not suitable for an appropriate comparison.

In marine phytoplankton, it has been proposed that during the final step in storage lipid biosynthesis (the formation of TAG), the diacylglycerol (DAG), which is a precursor of TAG, PL and GL, may follow PL synthesis to membrane lipids instead of TAG synthesis (Jónasdóttir 2019). If this is also the case in *C. crangon,* it would provide a reason for the low storage lipid (TAG) but high PL levels (Martínez-Alarcón et al. 2019b). It would, however, not explain the high number of transcripts of DGAT responsible for TAG synthesis (Table 6). Therefore, further analyses of the functionality of the DGAT sequences are required. Mutations that hamper functionality, as shown for the FABPs, would explain why DAGs are directed towards phospholipid synthesis instead of TAG synthesis.

5 Conclusions and outlook

Gene expression of digestive enzymes reflects the seasonal food availability and feeding activity of *C. crangon*. Elevated expression of phospholipase enzymes indicates utilization of intracellular lipid reserves, in this case polar lipids of biomembranes. The limited ability of *C. crangon* to store TAG remains unexplained. However, here we hypothesize that the mutation on the FABP binding cite could have some consequences for intracellular lipid transport and lipid storage. Furthermore, we found the relevant transcripts of those enzymes facilitating the respective anabolic pathways, i.e. the so-called Kennedy pathway. However, the number of transcripts was low compared to other enzymes and the functionality of the enzymes could not be evaluated due to limited bioinformatics and database entries and the small size of the

sequences. Future research will aim at elucidating the sequences, structures, and functionalities of the Kennedy pathway enzymes of *C. crangon* and other crustaceans with special focus on the key enzyme DGAT.

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Table 1. Origin and biological data of *Crangon crangon* samples

Date (2016)		Location		Ind. analysed (n)	Males (n)	Females (n)	Total length (mm)
February	19	54°08'N	007°52'E	10	2	8	55 - 70
April	18	53°44'N	008°15'E	10	2	8	53 - 70
July	19-21	53°44'N	008°15'E	10	5	5	55 - 68
October	24-26	54°08'N	007°52'E	10	2	8	62 - 74

Table 2. List of primers used for the gene expression analysis of the midgut gland of *C. crangon*

Category	Gene	Code	Forward primer (5´- 3´)	Reverse primer (5´- 3´)	Amplicon length (bp)
Housekeeper	**Actin**	Actin	CCGATAGTGATGACCTGACCG	TACCACTGCCGAGAGGGAAA	150
	HIF-1 alfa	HIF-1 alfa	CGCCGCTGACGATGTAATTG	TCAGGCCACTCTCATCAACG	77
	Hitone H3	His H3	AACAGACCGACAAGGTAGGC	TGGTACTGTTGCCCTTCGTG	183
Lipid metabolism	**Triacylglycerol lipase**	Tr_lip	CGTGGTACCCATCTTGCAGT	GGCATGGAATGGGAGACACA	219
	Phospholipase	Phos	TGGGTCATGTATCCCTCGTC	TTGGGGAGTTTGTGGCATTC	233
	Fatty acid desaturase	FAD	GCGTCTGTCTCGCTGTACTT	CTGGGTATCACCATGGGAGC	167
Protein metabolism	**Trypsin**	Try	AATGTCGTTGGAGCGTCTGT	GTGCTGCCCAGTGTTTTCAG	167
	Cathepsin L	Cat L	TTCATGCCATTCTCTTCGCC	CACCCAAGGGAAAACTCCAC	571
Carbohydrate metabolism	**Alpha amylase**	Al_am	AGAGTCGTCAGTCGGGTACA	TTGCTGGAACTCTGCGACAT	185
	Chitinase	Chit	TGCTTTGCCGGCAGATACAG	TGGGAATACCCTACTCAGCGA	132

Table 3. List of fatty acid binding protein (FABP) sequences used for the alignment with FABPs of *C. crangon* (NCBI ID: Reference sequence ID in the database of the National Center for Biotechnology Information) (ns: no specified).

Species	Tissue	Taxon	NCBI ID	Reference
Homo sapiens	adipocyte	Mammalia	NP_001433.1	Baxa et al. 1989
Homo sapiens	muscle	Mammalia	AAB02555.1	Peeters et al. 1991
Homo sapiens	muscle	Mammalia	CAA39889.1	Peeters et al. 1991
Homo sapiens	heart	Mammalia	NP_001307925.1	Börchers et al. 1990
Locusta migratoria	muscle	Insecta	AAB30739.1	Maatman et al. 1994
Drosophila melanogaster	ns	Insecta	NP_001027181.1	Matthews et al. 2015
Anopheles gambiae	ns	Insecta	Q17017	Holt et al. 2002
Schistocerca gregaria	muscle	Insecta	P41496	Price et al. 1992
Pacifastacus leniusculus	hemocyte	Malacostraca	NA	Söderhäll et al. 2006
Penaeus monodon	hemocyte	Malacostraca	NA	Söderhäll et al. 2006
Schistosoma japonicum	ns	Trematoda	AAG50052.1	Liu et al. 2004
Echinococcus granulosus	ns	Cestoda	AAK51437	unpubl.

Table 4. Stability ranking of candidate reference gene expression in the midgut gland of *C. crangon* by two different algorithms.

GeNorm			NormFinder		
Rank	**Gene**	**M**	**Rank**	**Gene**	**Stability**
1st + 2nd	Actin + HIF	0.691	1st	Actin	0.352
1st + 3rd	Actin + H3	0.768	2nd	HIF	0.434
			3rd	H3	0.467

Table 5. Correlation matrix of gene expressions of digestive enzymes in midgut glands of *C. crangon:* triacylglycerol lipase (Tr_lip), alpha amylase (Al_am), fatty acid desaturase (FAD), phospholipase A_2 (Phos), trypsin (Try), cathepsin L (CatL) and chitinase (Chit). P values marked red.

	Tr_lip	Al_am	FAD	Phos	Try	CatL	Chit
Tr_lip		0.21	0.05	-0.17	0.38	0.52	0.53
		(0.22)	(0.80)	(0.31)	(0.02)	(0.00)	(0.00)
Al_am			0.23	-0.20	0.32	0.38	0.44
			(0.17)	(0.24)	(0.05)	(0.02)	(0.01)
FAD				-0.29	0.05	0.04	0.15
				(0.09)	(0.78)	(0.81)	(0.39)
Phos					0.01	-0.13	-0.31
					(0.97)	(0.47)	(0.07)
Try						0.64	0.14
						(0.00)	(0.43)
CatL							0.26
							(0.12)
Chit							

Table 6. Proteins involved in lipid metabolism. Third line contains the number of transcripts found in the transcriptome of *C. crangon* midgut glands (Martínez-Alarcón et al. 2019a).

	Name of protein	Abbreviation	Number of transcripts (n)
Transport	Lipophorin	LPP	31
	Microsomal triacylglycerol transfer protein	MTP	4
	Fatty acid binding protein	FABP	18
	Non-specific lipid-transfer proteins	LTP	4
Anabolism	Glycerol-3-phosphate acyltransferase	GPAT	22
	Acylglycerophosphate acyltransferase	AGPAT	1
	Phosphatidic acid phosphohydrolase	PAP	2
	Diacylglycerol acyltransferase	DGAT	9
Catabolism	Triacylglycerol lipase	TAG lipase	14
	Phospholipase A2	PLA2	20

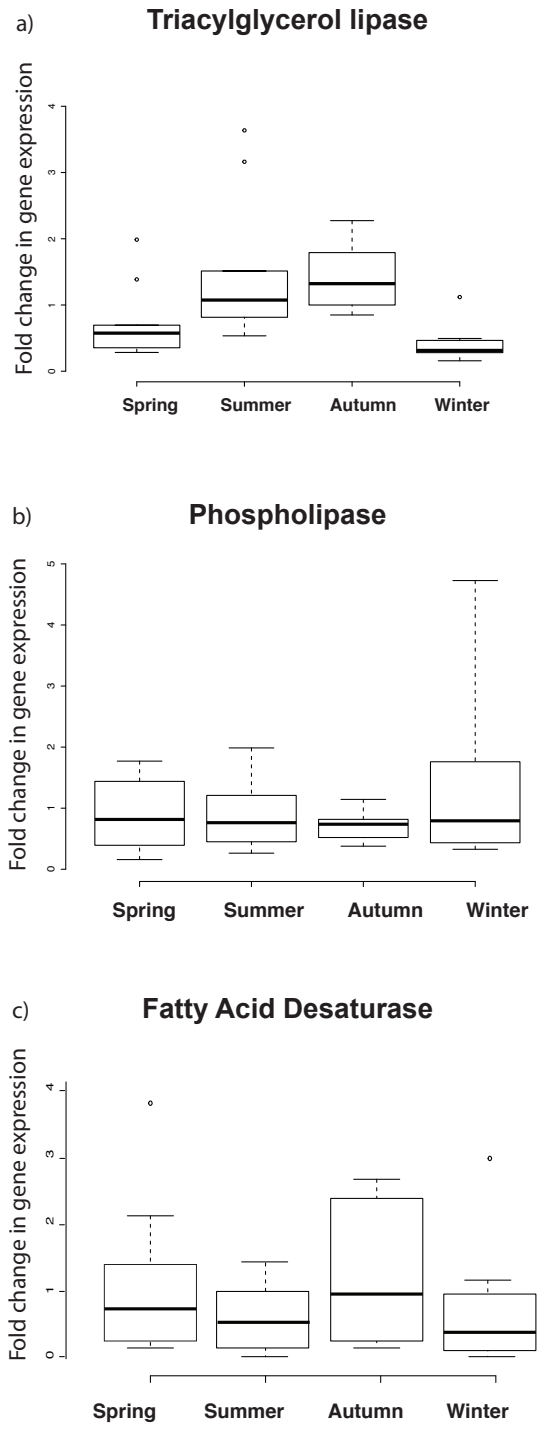

Fig. 1. Crangon crangon midgut glands: Seasonal regulation of digestive enzymes involved in lipid metabolism.

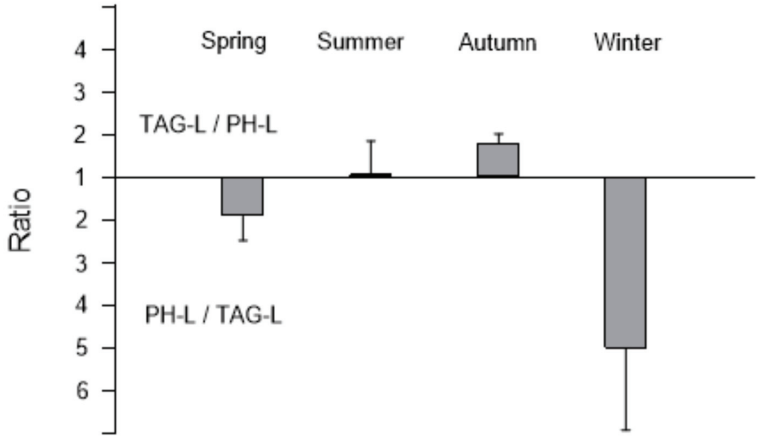

Fig. 2. *Crangon crangon* midgut glands: Seasonal ratio of gene expression of phospholipase and triacylglycerol lipase.

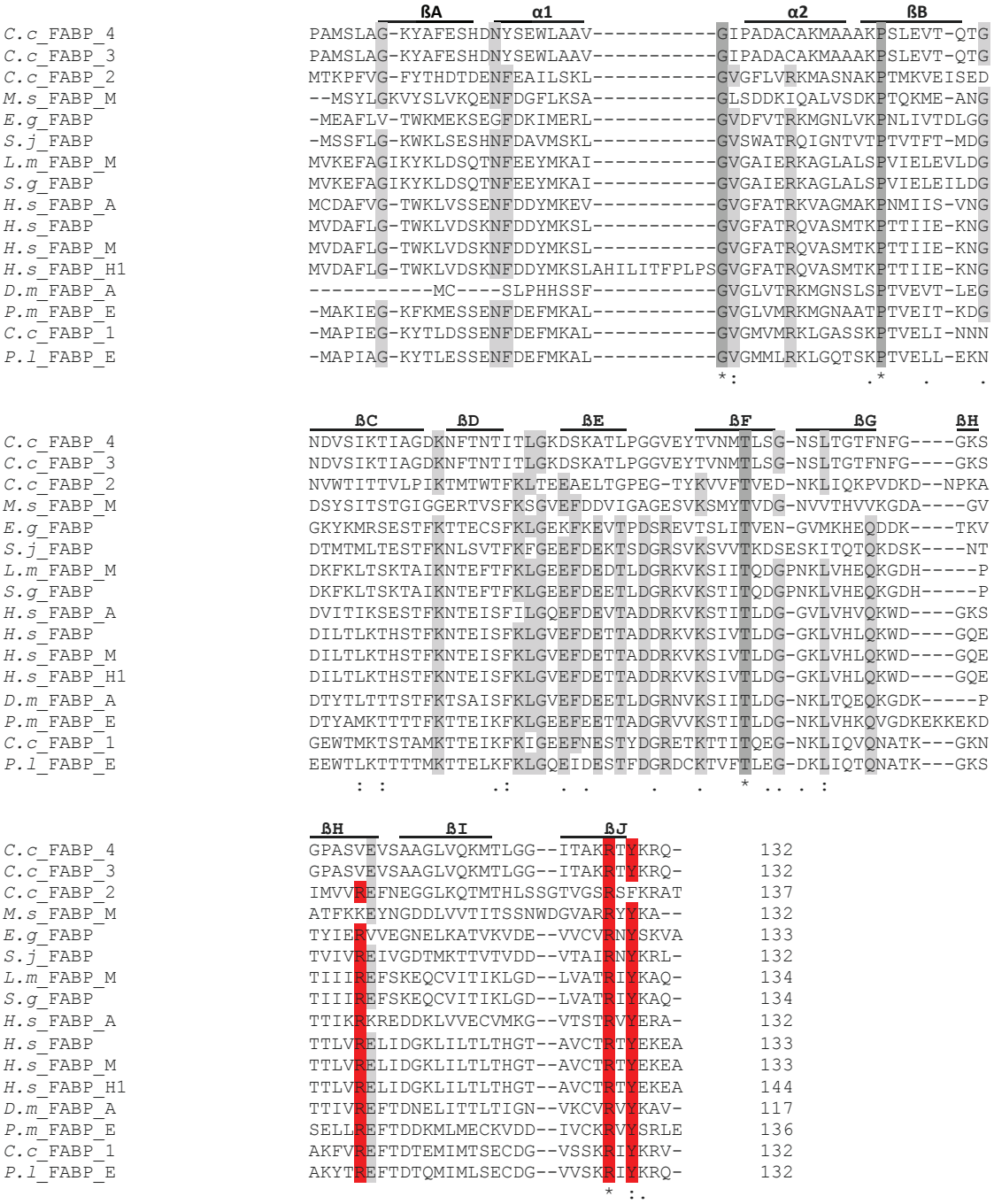

Fig. 3. Alignment of FABP sequences. Residues that are conserved 100% are highlighted in dark grey and residues conserved at least 75% in clear grey. The residues highlighted in red represent the three amino-acid residues (Arg110, Arg130, Tyr132) (numbers according to *Penaeus monodon* sequences) of the P2 motif characteristic of FABPs. The following sequences were aligned: *Crangon crangon* FABP isoforms 1 – 4 (C.c_FABP_1, C.c_FABP_2, C.c_FABP_3, C.c_FABP_4), *Schistosoma japonicum* FABP (S.j_FABP), *Locusta migratoria* FABP (L.m_FABP), *Drosophila melanogaster* FABP isoform A (D.m_FABP_A), *Homo sapiens* FABP from adipocyte (H.s_FABP_A), *Homo sapiens* FABP from muscle (H.s_FABP), *Homo sapiens* FABP from muscle (H.s_FABP_M), *Homo sapiens* FABP from heart isoform 1 (H.s_FABP_H1), *Echinococcus granulosus* FABP (E.g_FABP), *Anopheles gambiae* FABP (A.g_FABP), *Schistocerca gregaria* FABP (S.g_FABP) *Penaeus monodon* FABP from hemocytes (P.m_FABP_E) and *Pacifastacus leniusculus* from hemocytes (P.l_FABP_E). Secondary structure is indicated: α, α helix and β, β strands.

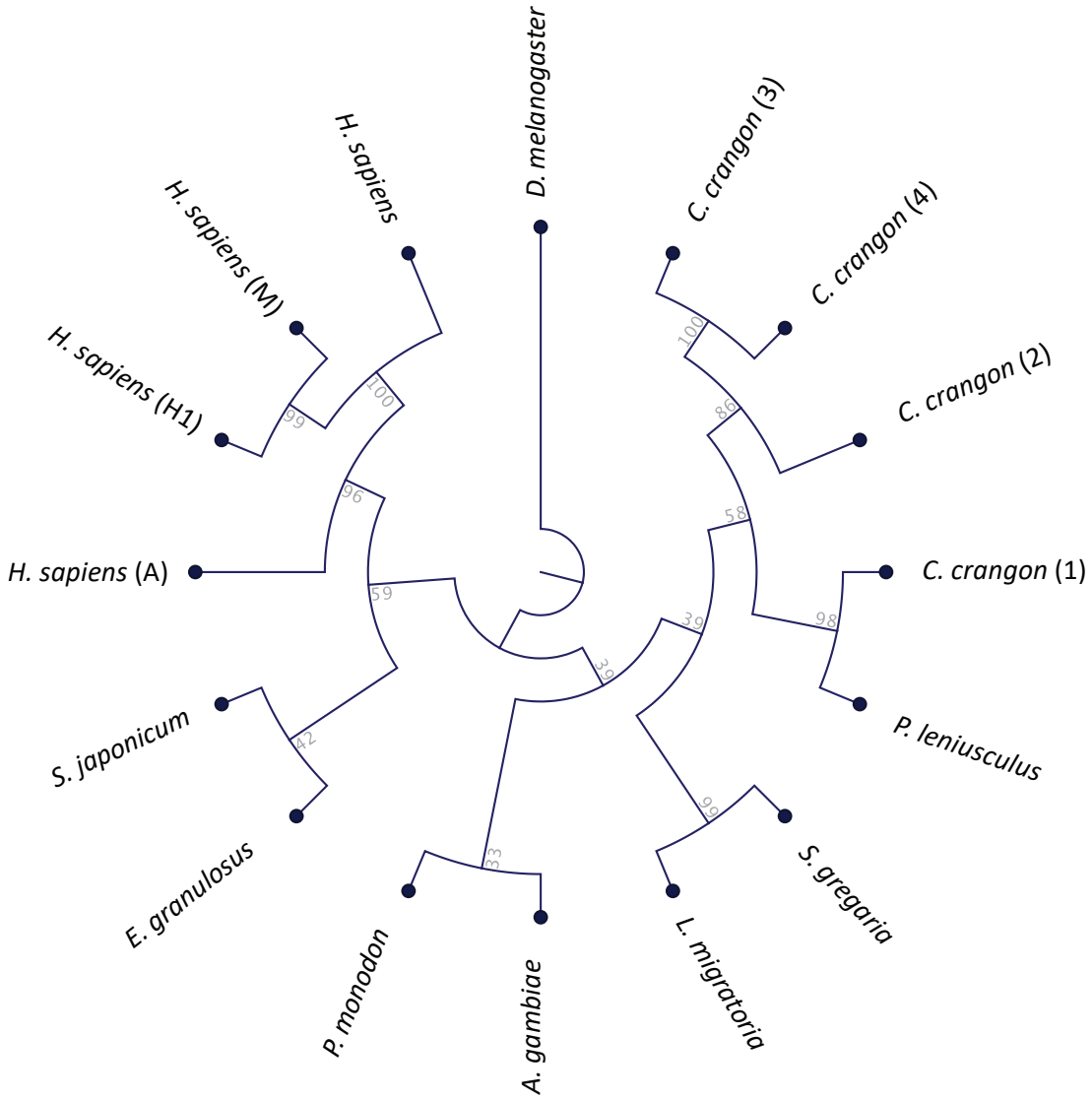

Fig. 4. Maximum likelihood cladogram of amino-acid sequences of fatty acid binding proteins of *Crangon crangon* (isoforms 1 to 4) and FABPs of other taxa from NCBI data. Bootstrap values are presented at the nodes. For abbreviations refer to Table 3.

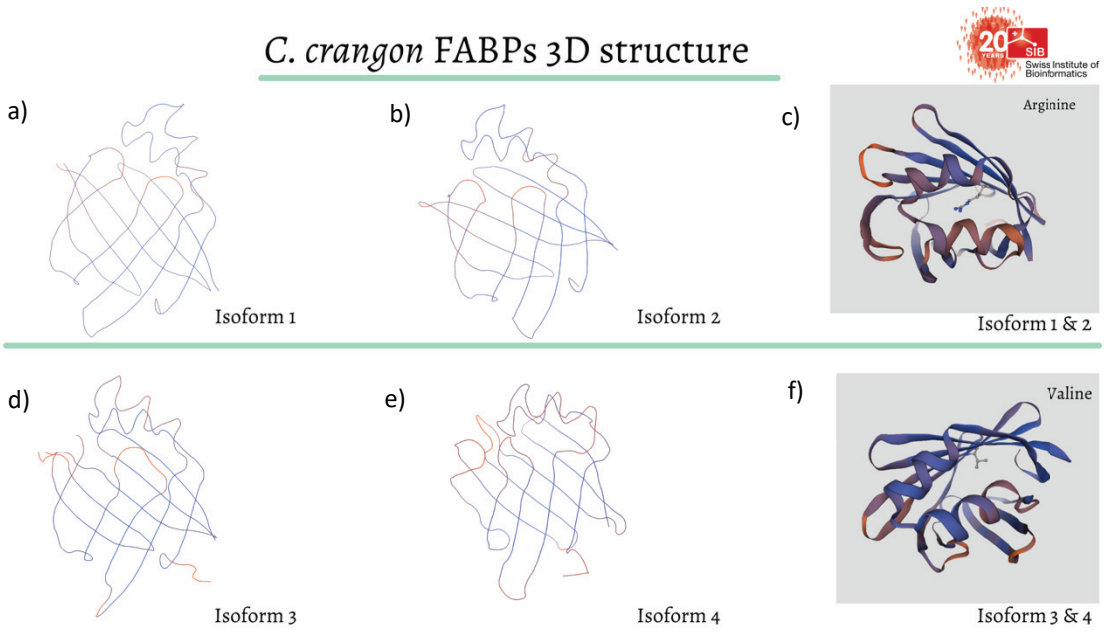

Fig. 5. 3D structure of the four isoforms from the midgut gland of *Crangon crangon*. Isoform 1 (a) and isoform 2 (b) have the three binding residues characteristic of the FABP family (Arg110, Arg130, Tyr132) (numbers according to *P. monodon* sequences). Representation of the arginine residue characteristic of the FABP family, which is present in isoforms 1 and 2 (c). Isoform 3 (d) and isoform 4 (e) have a valine residue instead of arginine. Representation of the valine residue, which substitutes the arginine residue in the isoforms 3 and 4 of *Crangon crangon* (f).

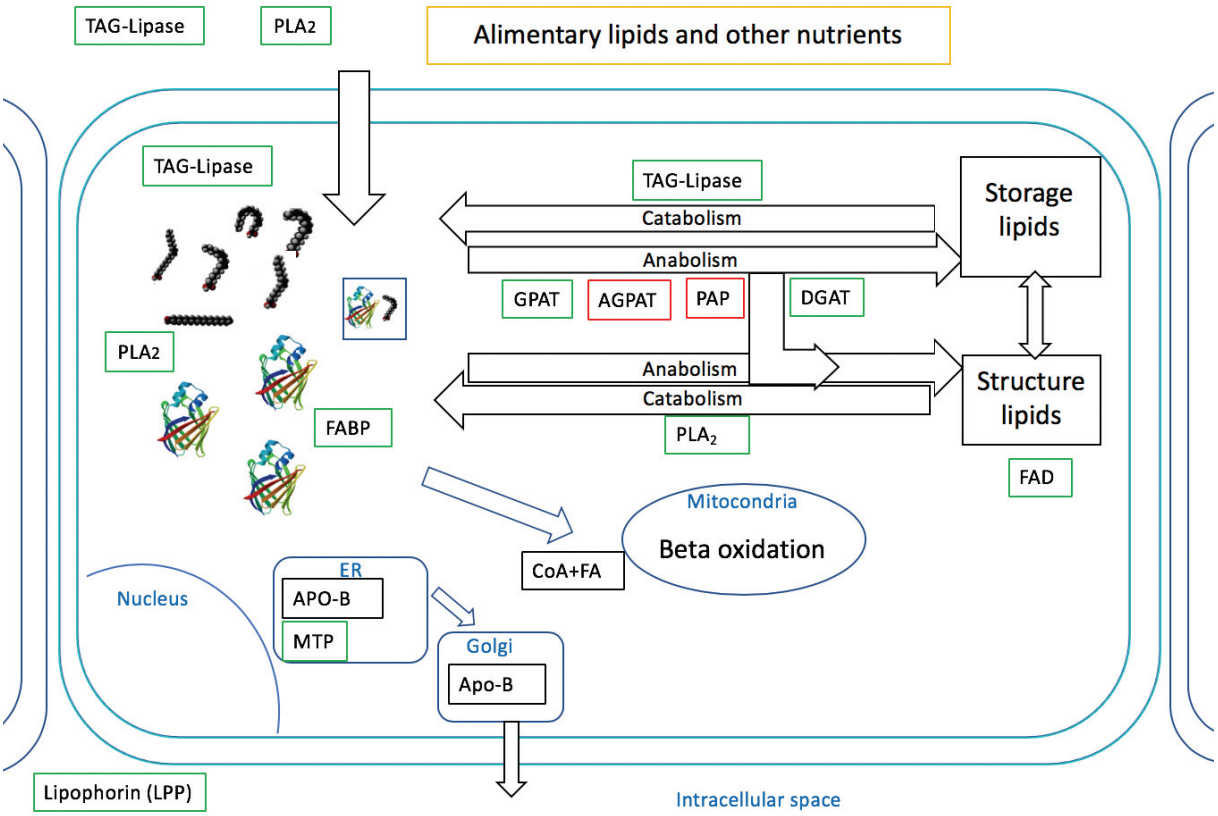

Fig. 6. Sketch of lipid metabolism in the midgut gland of *Crangon crangon*. Proteins and enzymes found in the transcriptome of *C. crangon* in green boxes. Proteins found in very low numbers in the transcriptome of *C. crangon* in red boxes. GPAT (glycerol-3-phosphate acyltransferase), AGPAT (acylglycerophosphate acyltransferase), PAP (phosphatidic acid phosphohydrolase), DGAT (diacylglycerol acyltransferase), MTP (microsomal triacylglycerol transfer protein).

CHAPTER 4

SYNOPTIC DISCUSSION AND PERSPECTIVES

The objective of this thesis was to investigate biochemical properties that support adaptive physiological processes in *Crangon crangon* in a variable and changing environment. Primarily, this study focused on the seasonal energy storage of *C. crangon* in comparison with the other North Sea shrimps *Crangon allmanni* and *Pandalus montagui*, with special emphasis on feeding preferences. Additional information obtained from the transcriptome of the midgut gland allowed studying the polymorphism of digestive enzymes in *C. crangon* and providing an explanation for the lipid storage strategies of *C. crangon*.

In addition to three scientific manuscripts (two already published) that are a product of this research, I included the sequence analysis of the digestive enzyme trypsin and the catalytic implications of a specific mutation in the catalytic site. The last part of this synoptic discussion includes the data of metabolites obtained from the midgut gland of *C. crangon*. All together, lipid, protein, transcript and metabolite data provide a much better understanding of the high degree of adaptive diversification in relation to the biochemical structures and processes of *C. crangon* in the North Sea.

4.1 Physiological aspects of lipid storage, feeding preferences and life cycle strategies of North Sea shrimps

Lipid storage and fatty acid composition of *C. crangon*, and *P. montagui* are included in the first publication of this thesis. In addition to the seasonal study of *C. crangon* and *P. montagui*, lipid storage and fatty acid compositions of *C. allmanni* were determined for July and compared with the data of *C. crangon* and *P. montagui*. No specimens of *C. allmanni* were available in February, April or October (Table 2), therefore a seasonal comparison was not possible. The absence of *C. allmanni* in February, April, and October could be explained by their seasonal migration, which is still not fully understood (Allen 1960; Creutzberg & van Leeuwen 1980; Blahudka &Türkay 2002). But *C. allmanni* could also prefer deeper areas and more oceanic conditions than the other shrimps sampled.

Table 2. Sampling time, location and biological data of *C. allmanni* (SND: sex not determined).

Date (2016)		Location		Ind. analysed (n)	Males (n)	Females (n)	Females without eggs (n)	Females with eggs (n)	SND (n)
July	19-21	53°44'N	008°15'E	14	5	7	5	2	2

During summer, the total lipid content (%DM) was significantly lower in the midgut glands of *C. allmanni* compared to those of *P. montagui* ($P < 0.0001$), but significantly higher than in those of *C. crangon* ($P < 0.001$). Total lipid levels in the midgut gland of *C. allmanni* ranged from 9% to 34%DM, in *C. crangon* from 7% to 19%DM and in the pink shrimp from 59%DM to 73%DM (Fig. 5).

Although the total lipid content in *C. allmanni* was higher than in the brown shrimp, it is still clearly below that of *P. montagui*. Apparently, to some extent *C. allmanni* also has low lipid storage capabilities. As suggested earlier, the limited lipid storage in *C. crangon* may be due to mutations in proteins involved in lipid metabolism (publication III). Therefore, the low lipid storage capability in *C. allmanni* may have the same molecular source and other crangonid species could share this molecular characteristic. This assumption is supported by other molecular characteristics that Crangonidae share, for example, the large size of the genome and the expression of cysteine instead of serine proteinases (Teschke & Saborowski 2005). However, this would need to be verified for the other crangonid species not investigated yet.

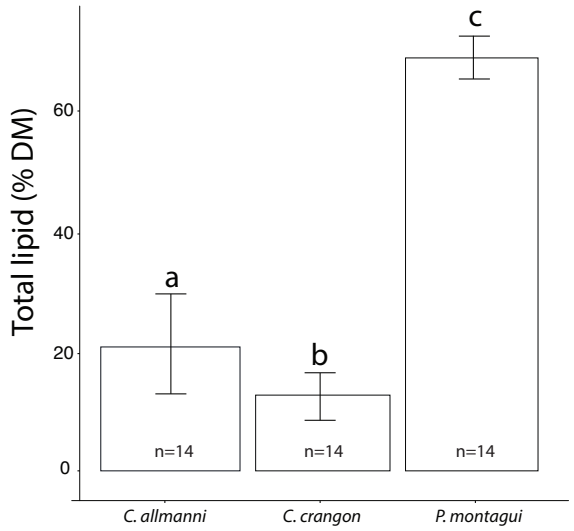

Fig. 5. Total lipid contents (% of dry mass, DM) of the midgut glands of *Crangon allmanni*, *Crangon crangon* and *Pandalus montagui* from July 2016. Error bars represent standard deviations. Different letters (a, b, c) indicate significantly different values ($p < 0.001$).

According to the trophic marker concept, fatty acids in lipid depots can provide information about trophic preferences (Dalsgaard et al. 2003). However, due to the rather low lipid level in the midgut gland of *C. allmanni* during summer, all assumptions regarding its feeding preferences should be taken with caution. The fatty acid compositions of *C. allmanni* show similarities with *C. crangon,* but also with *P. montagui*. The carnivory:herbivory index of *C. allmanni* was closer to that of *P. montagui* than that of *C. crangon*, but the diatom ratio was closer to *C. crangon* than *P. montagui* (Table 3).

Table 3. Fatty acid composition of the midgut gland of *Crangon allmanni* expressed as percentage of total fatty acids (% TFA). The sum of herbivory markers (%) (16:1(n-7) and 18:1(n-7)), diatom ratio (16:1(n-7)/16:0), carnivory/herbivory index (18:1(n-9)/ Σherb.+18:1(n-9)) are also presented. Values are given as means ± SD. n= number of samples analyzed. Concentrations <1% TFA are not presented.

Species	*C. allmanni*		
Season	Summer		
	n=14		
Fatty acids (%)			
14:0	3.8	±	1.5
16:0	13.7	±	1.5
16:1(n-7)	7.4	±	3.3
iso 17:0	1.6	±	0.4
16:3(n-4)	1.4	±	0.9
18:0	5.0	±	1.4
18:1(n-9)	6.7	±	2.3
18:1(n-7)	7.1	±	0.6
20:1(n-11)	3.1	±	1.3
20:1(n-7)	2.6	±	1.3
20:4(n-6)	3.3	±	1.8
20:5(n-3)	17.8	±	3.4
22:5(n-3)	3.1	±	1.5
22:6(n-3)	12.4	±	2.1
Σsaturated	24.1	±	0.8
Σmonounsaturated	26.9	±	0.6
Σpolyunsaturated	37.9	±	0.5
Sum of herbivory markers (%)	14.5	±	3.4
Diatom ratio	0.5	±	0.2
Carnivory:herbivory index	0.3	±	0.1

Apparently, *C. allmanni* shows similar lipid storage characteristics as *C. crangon*, at least in quantitive terms during summer. It is difficult to assess, whether or not the lipid storage in *C. allmanni* is linked to their reproductive effort. It has been suggested that in the Crangonidae

there is a great similarity in the general features of larval development, especially between the congeners *C. crangon* and *C. allmanni* (Criales & Anger 1986). However, according to Blahudka & Türkay (2002), *C. allmanni* has a prolonged breeding season, which probably includes one or two spawning events and recruitments. Since the reproductive effort of *C. allmanni* is much lower than that of *C. crangon* (Clarke 1979b; Blahudka & Türkay 2002), *C. allmanni* may require less lipid for reproductive processes than *C. crangon*.

The similar results for *C. allmanni* support the idea that the limited lipid storage in *C. crangon* may have a genetic source. To clarify if the midgut gland of *C. allmanni* also functions as a metabolic center rather than a storage organ, additional lipid data from other seasons are necessary, as well as detailed analyses on proteins involved in the lipid metabolism.

This thesis presents data on the seasonal lipid storage in the midgut gland of *C. crangon* and *P. montagui,* and the lipid storage of *C. allmanni* during summer. Based on this information it can be concluded that *C. crangon* utilizes the midgut gland as a metabolic center rather than a storage organ, while *P. montagui* relies more on the energy storage function of the midgut gland. Furthermore, seasonal variation in lipid storage and the lipid class composition of the midgut gland of *C. crangon* and *P. montagui* suggested that lipid levels in *P. montagui* are linked to the reproductive cycle, which is not the case in *C. crangon*. Applying the fatty acid trophic marker concept, feeding preferences could be suggested for *P. montagui*, but dietary signals were not very clear for *C. crangon* and *C. allmanni*, due to the lower lipid levels. *C. crangon* seems to have a broader dietary spectrum than the other two North Sea shrimps. All this information suggests that *C. crangon* does not rely on its lipid depots to cope with a variable environment, where the food supply changes constantly. Instead, the life strategies of the brown shrimp depend more on an opportunistic feeding behaviour and the dynamic metabolic activities of their midgut gland.

4.2 Implications of the genome size on evolution and reproduction of *C. crangon*

Genome size has a major impact on growth, metabolism and life-history traits in arthropods (Alfsnes et al. 2017). In Crustacea, genome sizes tend to be highly variable among families, particularly within the order Decapoda (Gregory 2002). The genome sizes of the vast majority of the species in this order range between one and five picograms of DNA content in a haploid cell (C value). Interestingly, according to the database *Animal Genome Size*, all Crangonidae have genomes larger than 9.9 (C value) (Fig. 6). *C. crangon* is not an exception with a genome size of 11.4 (C value), while the pink shrimp *P. montagui* has a genome size of 8.5 (C value). In

crustaceans, the weak association between genome size and phylogeny suggests that life cycle strategies and habitats are important determinants of genome size (Alfsnes et al. 2017). Living in a colder environment with low developmental rates may be one of the many promoters of large genomes in marine crustaceans. Also, invertebrates with larger genomes tend to exhibit broader ecological tolerance compared to those with smaller genomes (Cavalier-Smith 1978; Hardie & Hebert 2004; Alfsnes et al. 2017).

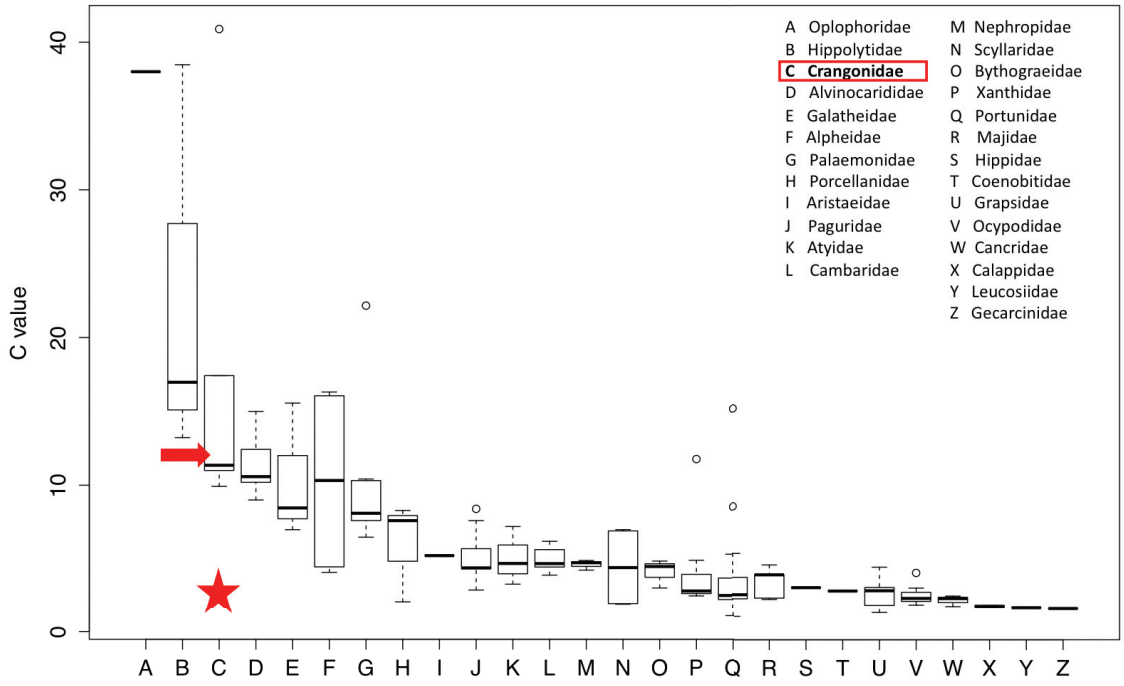

Fig. 6. Genome size of the different families of Decapoda (letters A-Z) reported in the Animal Genome Size Database. The C value represents picograms of DNA content in a single haploid cell. C value of *Crangon crangon* (11.4) indicated by red arrow. For comparison, the C value of *Homo sapiens* (3.5) is presented by a red star below the Crangonidae. (This graphic was produced with the software R based on data from the Animal Genome Size Database).

In some vertebrates such as fish and amphibians, genome size is positively correlated with the diameter of the eggs (Hardie & Hebert 2004), and the cell size (Cavalier-Smith 1978; Gregory 2002; Levy & Heald 2015), but negatively correlated with developmental rates (Horner & Macgregor 1983; Gregory 2002). According to the latter authors, the larger the genome is, the larger the cell and the longer the cell cycle time. Cell size and cycle time are important for development, as well as in some reproductive processes, e.g. gamete production. Therefore, the size of the genome has an impact on developmental rates.

But it does not apply to the crustacean species here studied. *C. crangon* with bigger genome has smaller eggs than those of *P. montagui*. Additionally, *C. crangon* brood eggs for eight to ten

weeks (clutch size of 1,000-14,000 eggs), while in *P. montagui* this process takes four to five months (clutch size of 150-4,000 eggs) (Clarke 1979b). Even if the developmental rate of *C. crangon* (Hünerlage et al. 2019) and *P. montagui* (Clarke 1979a) is related to their ambient water temperatures, *C. crangon* seems to show a faster development than with *P. montagui*.

Larger genomes in K-selected decapod species in comparison to smaller genomes in r-selected decapod species suggest a link between genome size and life-history characteristics such as development rate (Gregory 2002; Rees et al. 2008). Obviously, this observation is not valid for *C. crangon*, since the brown shrimp is an r-selected species with a large genome size. It would be interesting to clarify in a future study, why *C. crangon* with large genome size and low lipid storage has a faster developmental rate and bigger clutch size than *P. montagui*, which has a smaller genome size and more lipid reserves.

4.3 Source of digestive enzyme polymorphism in *C. crangon*

The variability of selected digestive enzymes was assessed through putative isoforms in the transcriptome of the midgut gland of *C. crangon.* As observed in the activity of serine endopeptidases (Saborowski et al. 2012), the transcripts also show a high level of polymorphism (publication II). The reason for this pronounced polymorphism is still unknown, however, in this thesis it is suggested that the potential to express different digestive enzyme isoforms improves the efficiency of *C. crangon* to utilize energy from a variety of food sources. This characteristic may be seen as an advantage, especially when living in variable environments, where food availability is frequently changing.

The evolutionary significance of enzyme isoforms is still not fully understood. Enzyme isoforms can differ in substrate affinity and reaction rates. Many studies related to enzyme polymorphism have been published, trying to elucidate the source of enzyme polymorphism and the consequences for the fitness of the individual (e.g. Lee 1984; Boucher et al. 2016). A relation between genetic polymorphism and environmental diversity (Powell 1971; McDonald & Ayala 1974), and feeding preferences has been documented (Van Wormhoudt et al. 1995; Le Moullac et al. 1996; Sellos et al. 1998). A recent hypothesis states that selection may favour isoforms, because they can increase the phenotypic robustness of the system (Tomaiuolo et al. 2008). However, this is a very complex topic, because many variables have to be taken into consideration. Each enzyme polymorphism has its own characteristics in terms of source and

function and they also depend on the different tissues in various organisms. Therefore, it is not possible to establish a general rule that applies to all types of enzyme polymorphism.

Enzyme isoforms can be products of different processes. One of them is the duplication of genes, which both remain in the same genome (Magadum et al. 2013). Gene duplication can have different fates: (I) It can result in the loss of a function, (II) it can develop a new function, or (III) both genes become complementary of each other and therefore both copies are necessary for the system (Force et al. 1999). From an energetic point of view, gene duplication is the most expensive source of polymorphism, because the transcription regulation requires high energetic costs and consumes time. To obtain a functional protein it takes from minutes to hours, depending on cell turnover rates and gene size (Pérez-Ortin et al. 2007), time needed for gene transcription and transcriptional regulation.

Another source of enzyme isoforms is the alternative splicing of genes (Fig. 7), in which different combinations of exons produce different mRNA isoforms of the same gene (Gilbert 1978; Modrek & Lee 2002). Alternative splicing is one of the main sources of proteomic diversity in multicellular eukaryotes (Nilsen & Graveley 2010). The number of mRNA isoforms that a single gene can encode varies from two to several thousands (Nilsen & Graveley 2010). In some mammalian tissues, the high degree of polymorphism originating from alternative splicing correlates with high organismal complexity (Nilsen & Graveley 2010). The biochemical mechanisms that are involved and control alternative splicing are complex and poorly understood. However, it is known that some alternative splicing events are regulated. This has been deduced from distinct splicing patterns observed in different tissues and different developmental stages in various eukaryotes including arthropods (Nilsen & Graveley 2010).

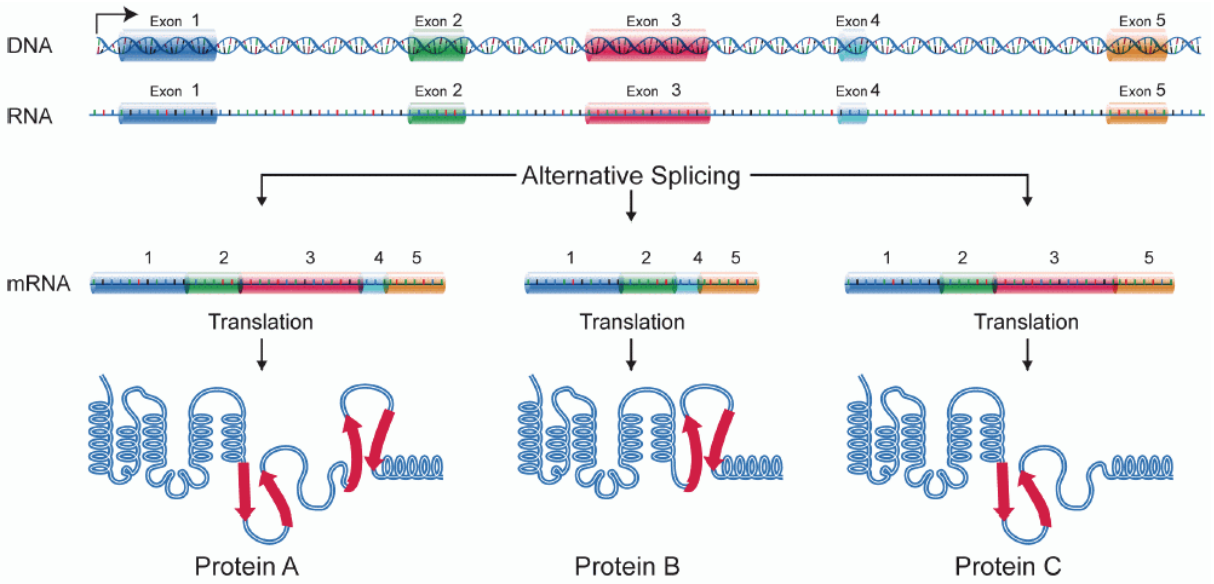

Fig. 7. Example of alternative splicing. Gene splicing is done in eukaryotes, prior to mRNA translation by the differential inclusion or exclusion of regions of pre-mRNA. The mRNA molecules are translated to different proteins or isoforms of the same protein. (Credit: National Human Genome Research Institute).

In the case of *C. crangon,* the source of the high polymorphism in the midgut gland is still unclear. Since the midgut gland shows a limited lipid storage and high metabolic demand, the required energy could be a rate-limiting factor. In large genomes like the one of the brown shrimp, gene transcription may take longer. In this context, it could be convenient for the brown shrimp to use alternative splicing as a source of the enzyme polymorphism of the midgut gland. The regulation of isoenzymes by alternative splicing requires modifications at the post-transcriptional level but not at the genetic level. This could be an inexpensive way of increasing the robustness of the system (Tomaiuolo et al. 2008). In the transcriptome of the midgut gland of *C. crangon* five transcripts were identified that are involved in alternative splicing events. However, it is not possible to determine, if these isoforms are related to those alternative splicing transcripts or if they are the product of gene duplication.

Isoform proportions of digestive enzymes in *C. crangon* could be dynamically regulated by expressing isoforms with more or less affinity to specific substrates according to specific needs. According to Tomaiuolo et al. (2008) dynamic regulation of isoform proportions by alternative splicing seems to be biologically plausible. In response to environmental stress, alternative splicing is a fast mechanism for the regulation of gene expression (Colot et al. 2005; Pleiss et al. 2007). In this way, alternative splicing may be a potent and metabolically inexpensive way to increase system robustness (Tomaiuolo et al. 2008).

4.4 Mutation in the catalytic triad of trypsin in *C. crangon*

To better understand, how well the brown shrimp is adapted to a dynamic environment, I focused on those molecular particularities that are unique to *C. crangon*. The substitution of serine proteinases for cysteine proteinases in *C. crangon* (Teschke & Saborowski 2005) appears to be a key characteristic that can reveal more information about the molecular processes in *C. crangon*. In decapods, serine proteinases are in charge of the major part of the protein digestion, and trypsin is one of the main serine proteinases in the midgut gland of decapods. However, in *C. crangon* trypsin participation is not as distinct as in other decapods. Therefore, the structure of this enzyme was analysed to elucidate the reason for the low trypsin activity in the midgut gland of most *C. crangon* individuals.

Trypsin is synthesized as a pre-proenzyme and stored as the proenzyme trypsinogen. After release into the gut, trypsinogen is activated by other enzymes. Once activated, the trypsin contributes to the digestion of ingested protein, but also to the activation of other digestive enzymes such as chymotrypsin and elastase (Baird & Craik 2013). The catalytic triad of trypsin is composed of His 57, Asp 102 and Ser 195 (Baird & Craik 2013) (Fig. 8).

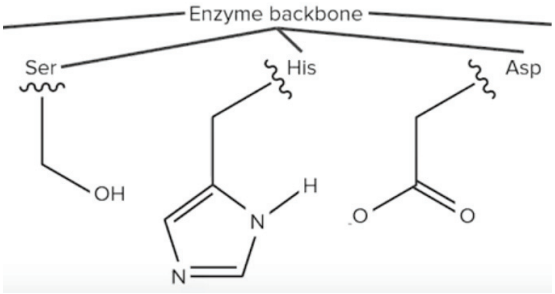

Fig. 8. Schematic illustration of the active site in the serine protease trypsin. Histidine (His) acts as a base catalyst to activate Serine (Ser), Ser acts as nucleophile and aspartic acid (Asp) stabilizes protonated His.

Trypsin sequences produced by the gene expression analysis from midgut glands of 32 *C. crangon* individuals were analyzed from four different seasons. The sequences were translated to their respective amino acids and compared with other trypsins previously reported. Surprisingly, the majority of the trypsin synthesized in the midgut gland of *C. crangon* shows a mutation in the histidine (His) 57 of the catalytic triad. Instead of a (His) the sequences have a glutamine (Gln) (Fig. 9). Previously, it was reported that the modification in a member of the catalytic triad has resulted in a dramatic loss of activity (Hehemann et al. 2008; Baird & Craik 2013). The mutation of (His) 57 at the catalytic site of trypsin suggests that the trypsin in

the midgut gland of *C. crangon* is not fully functional or it has a reduced activity, since the amino acid that is substituting (His) has a different chemical characteristic. Histidine is an amino acid with an electrically positively charged side chain, whereas glutamine has an uncharged polar side chain (Fig. 10). This has major catalytic implications for trypsin, because the positive charge of histidine is necessary to start the catalytic reaction.

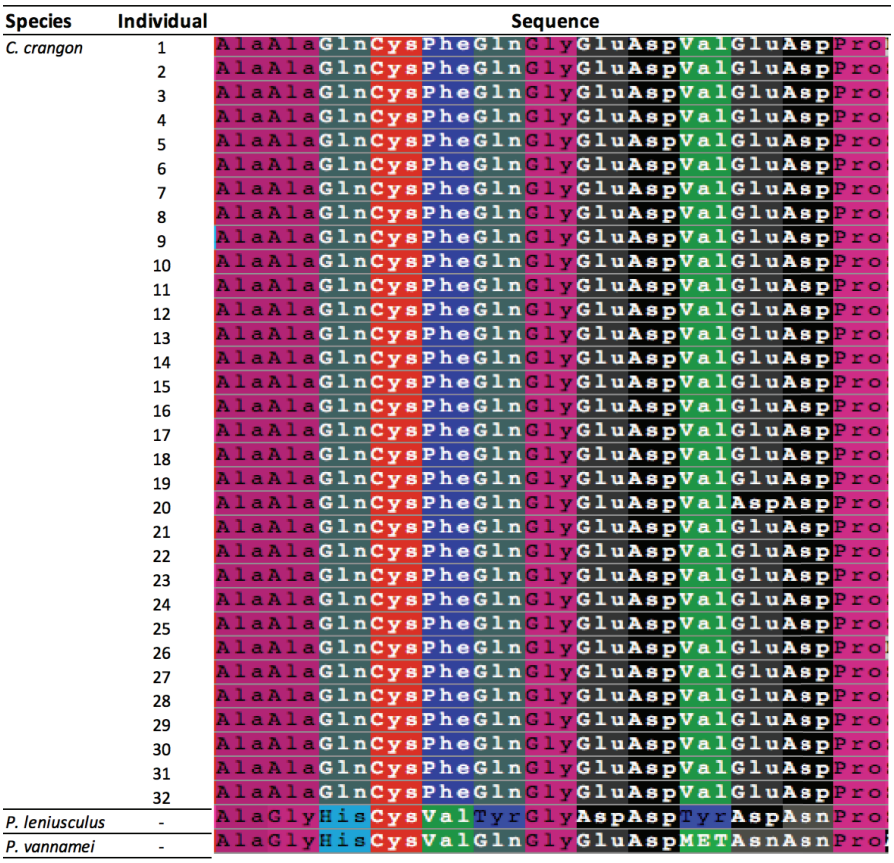

Fig. 9. Alignment of trypsin sequences of *C. crangon* with sequences of *Pacifastacus leniusculus* and *Penaeus vannamei*. 32 sequences of 32 *C. crangon* specimens.

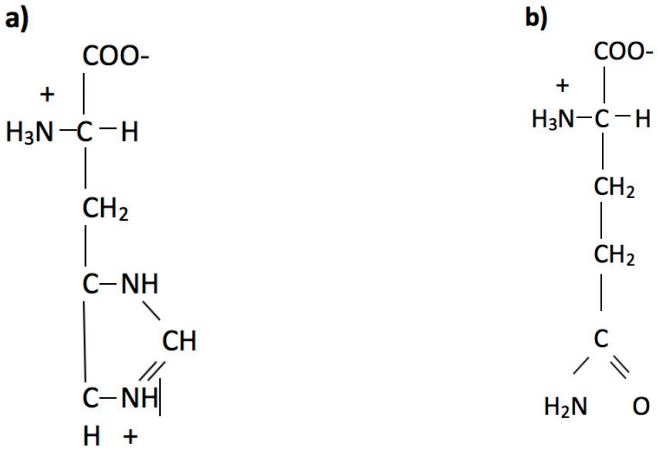

Fig. 10. Chemical structure of the amino acids histidine (His) and glutamine (Gln).

This mutation may explain why *C. crangon,* contrary to most decapods, uses cysteine instead of serine proteinases (Teschke & Saborowski 2005). The use of cysteine instead of serine proteinase has also been reported in the caridean *Crangon allmanni,* (Teschke & Saborowski 2005), but no information is available about its protein sequence. The trypsin sequence with the same amino acid substitution as in *C. crangon* also occurred in the giant freshwater prawn *Macrobrachium rosenbergii* (Cheng et al. 2014), but there is no information about its activity.

In addition to the analysis of sequences that are the product of gene expression, trypsin sequences obtained from the transcriptome of the midgut gland of *C. crangon* (publication II) were also analyzed. All sequences of the gene expression analysis showed the above mutation, but not all sequences obtained in the transcriptome showed this mutation. This contrasting result indicates that *C. crangon* has isoforms with and without the mutation, but they express mostly the one with the mutation. The sequences of the gene expression analysis were from specimens of all seasons and all showed the mutation. Therefore, we can conclude that the expression of isoforms with mutation is independent of the season of the year.

This mutation could explain the low trypsin activity in the midgut gland of the brown shrimp. However, it is still not clear if trypsin activity is negatively affected by this mutation and what implications it really has on the performance of *C. crangon*. In publication II we suggested that the degree of polymorphism in *C. crangon* is related to the function of the enzymes. But as mentioned previously in this discussion, the expression of isoforms is energy-consuming.

Therefore, it is an open question, why *C. crangon* shrimps conserve and express enzymes that may have no catalytic power.

4.5 Metabolites in the midgut gland of *C. crangon*

Metabolites are intermediate or final products of biochemical pathways in living systems. Metabolites are essential to growth and life processes. In some cases, alterations in metabolite concentrations result from adaptations to different stressors (Tappan et al. 1957; Paschke et al. 2010). Physiological stress can generally be compensated by an increase in other physiological responses. In *Cancer magister* (Dana, 1852) the change in metabolite concentrations in the digestive gland suggests an advantageous reaction to inhabit temporarily low oxygen zones (Paschke et al. 2010). In the case of *C. crangon* those individuals with low trypsin activity may experience stress with consequences in the modulation of some metabolites. As shown in the metabolic response of mammals (Tappan et al. 1957), mollusks (Tripp et al. 2017) and crustaceans (Paschke et al. 2010) under external stress.

Metabolites in the midgut gland of *C. crangon* were analyzed with two different objectives. The first one was to obtain a profile of metabolites of the midgut gland of *C. crangon*, which was unknown until now. The second objective was to compare the metabolic profile of individuals with high and low trypsin activity. We hypothesized that those specimens with high trypsin activity (ca. 10% of the population) may use different metabolic pathways than the rest of the population with low or medium trypsin activities. To test our hypothesis, we analyzed around 100 midgut glands of *C. crangon* for trypsin activity. From those, we selected five individuals each with low, medium and high trypsin activity.

Thirty-two metabolites were identified including free amino acids (glutamate, glutamine, dimethylglycine, alanine, threonine, tyrosine, phenylalanine, valine, isoleucine, leucine, methionine, glycine, beta-alanine), organic osmolytes (homarine, betaine, taurine), energy-related compounds (succinate, lactate, acetate), and others (trigonelline, snGlycero3phosphoc, dimethylamine, trimethylamine, fumarate, creatine, sarcosine, triphosphates, o-phosphocholine, choline, TMAO, creatinine, and malonate). Heat maps were constructed to visualize changes in metabolite concentrations in the three groups (high, medium and low trypsin activity). However, changes in the concentrations of metabolites could not be related to a specific group (Fig. 11).

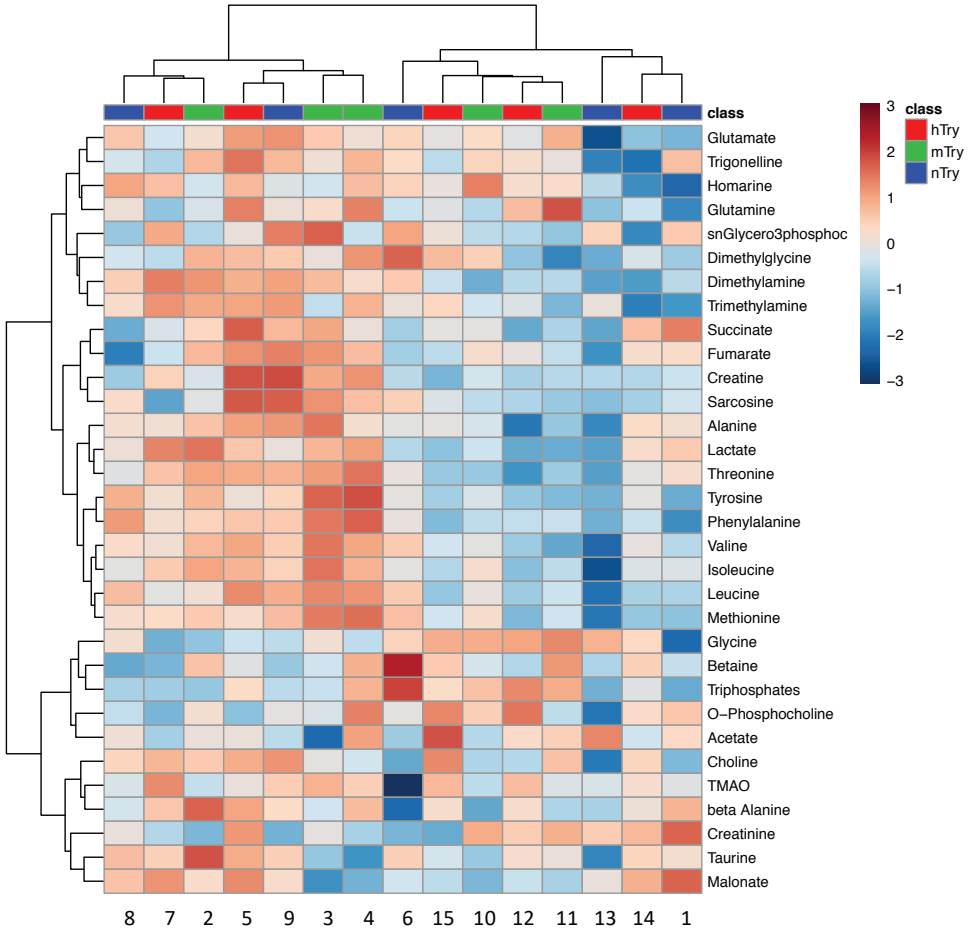

Fig. 11. Heatmap with scaled and centered concentrations values obtained from metabolite profiles of *Crangon crangon* midgut gland extracts. Individuals (#5,7,12,14,15) with high trypsin activity (hTry), individuals (#2,3,4,10,11) with medium trypsin activity (mTry) and individuals (#1,6,8,9,13) with low trypsin activity (nTry). Dark red values indicate higher concentrations and blue values indicate lower concentrations of the metabolites.

The three groups were compared separately for each metabolite (Fig. 12). However, there were no significant statistical differences detected for each metabolite between the three groups. These results indicate that metabolite concentrations are not related to the level of trypsin activity in the midgut gland of *C. crangon*. However, it is important to take into consideration that the number of replicates used in this study was relatively low (five in each group). This analysis could be improved by increasing the numbers of samples and differentiating the individuals by gender as well as developmental and maturity stage. In crustaceans, the utilization of different metabolic pathways may vary in a single individual according to the moulting stage (Lockwood 1968). For this analysis, individuals were not separated by gender or developmental stage.

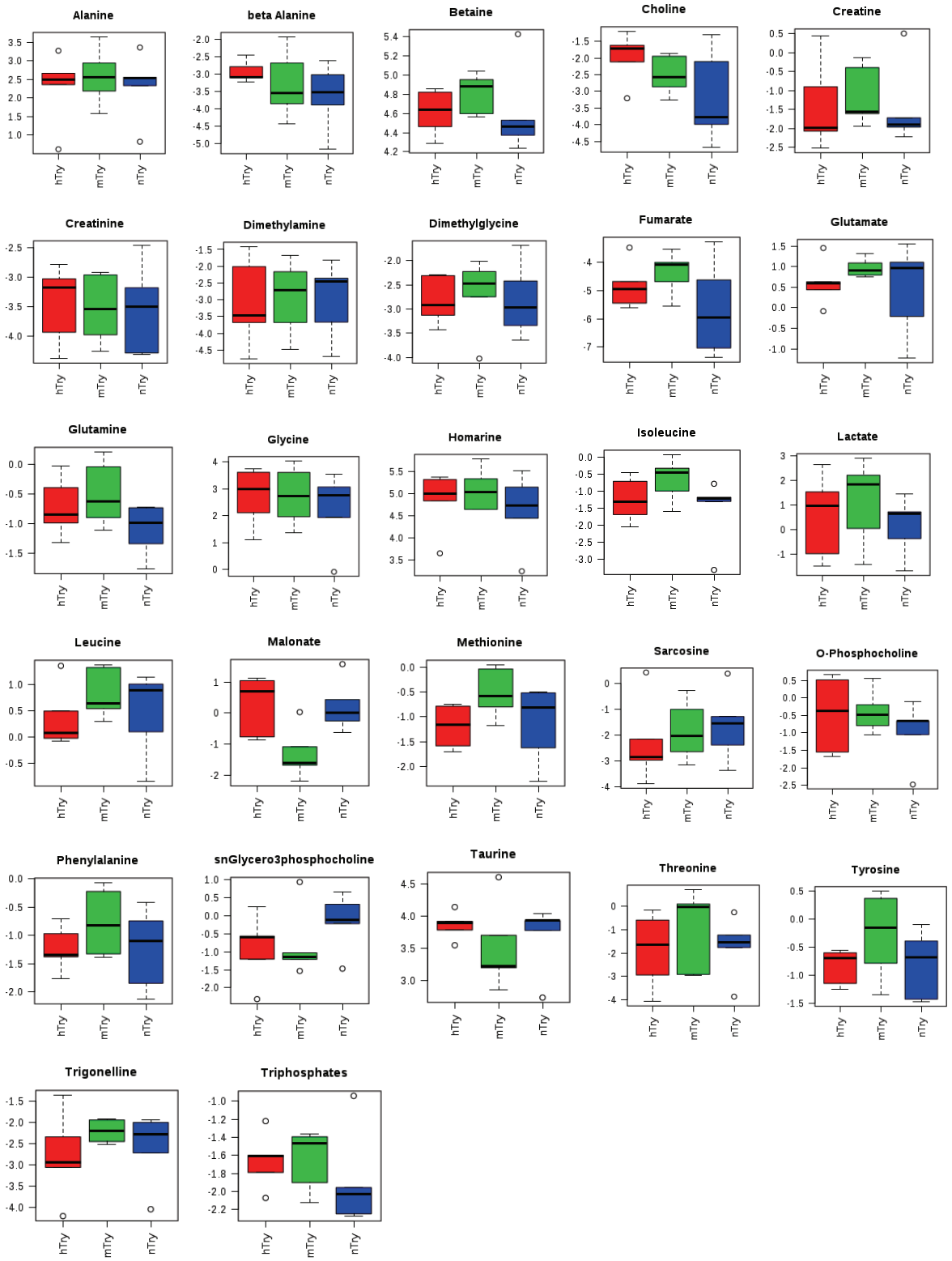

Fig. 12. *C. crangon* midgut glands: Comparison of specific compounds in the three groups testing individuals with high (hTry), medium (mTry) and low trypsin activity (nTry).

A considerable fraction of the determined metabolites are free amino acids (Fig. 11), which can have different sources. Their concentrations may vary in different tissues of the same individual (Mente et al. 2010), and they can play an important role as intracellular osmotic

effector (Huong et al. 2001; Intanai et al. 2009; Mente et al. 2010). The free amino acids arginine, alanine, aspartate, serine, glutamate, proline, and glycine have been detected in the midgut glands of the shore crab *Carcinus maenas* (D' Aniello 1980) and the spider crab *Maja verrucosa* (D' Aniello 1980). Within this research, a second heatmap was generated to elucidate relationships between metabolites (Fig. 13). The results show significant relationships between the amino acids (glutamate, dimethylglycine, alanine, threonine, tyrosine, phenylalanine, valine, isoleucine, leucine, methionine, beta-alanine), except for glycine, which is more related to triphosphates. Furthermore, the energy-associated compounds succinate and lactate are strongly related, but there is no relationship of either of the two with acetate, also an energy-related compound. Finally, the strong relationship of fumarate to succinate may be due to their close association in the Krebs cycle. However, to obtain conclusive results about the modulation of these compounds in response to energy demand, it would be necessary to carry out well-designed experiments with animals exposed to different stress conditions.

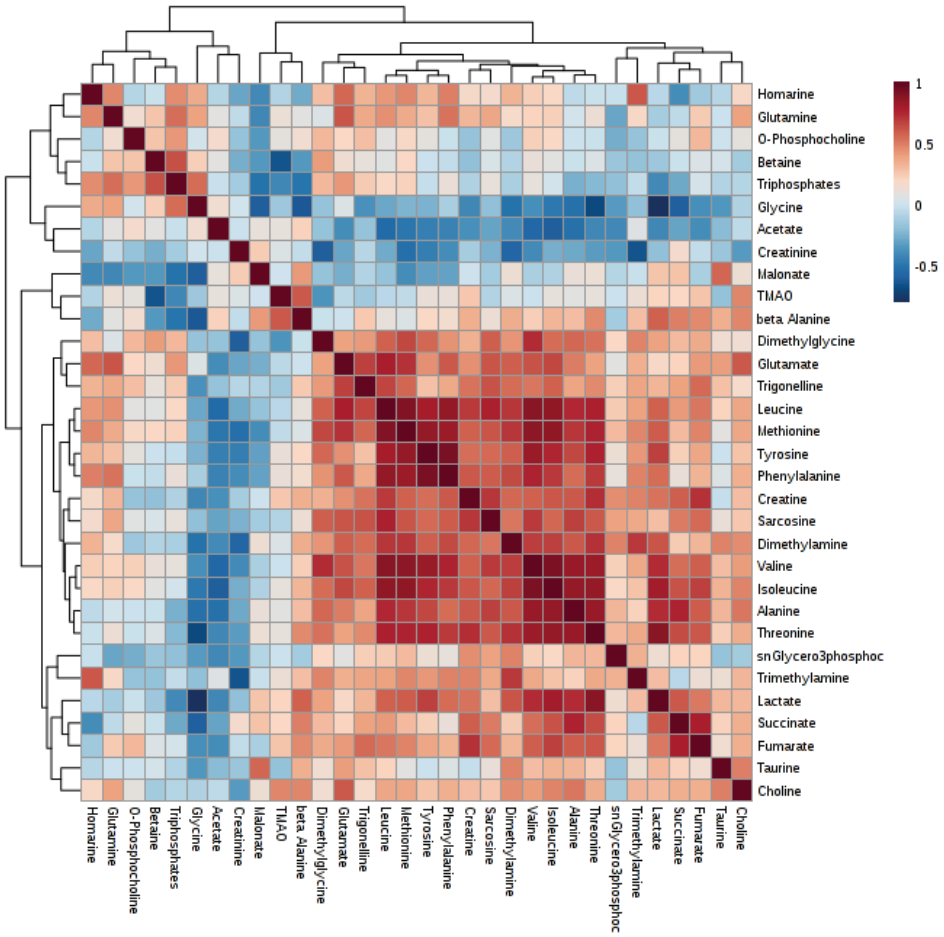

Fig. 13. Heatmap with scaled concentrations of values obtained from metabolite profiles of *Crangon crangon* midgut gland extracts. Red boxes indicate a strong relationship between metabolites.

The analysis of metabolites from the midgut glands of *C. crangon* provides a metabolic profile of this species and it revealed specific relationships between compounds. This analysis also showed that trypsin activity in the midgut gland of brown shrimps is not related to metabolite concentrations. Therefore, we can conclude that the brown shrimp is not under stress due the lack of trypsin activity. Further analysis is necessary to clarify differences with regard to gender and developmental stages. To elucidate adaptive strategies of *C. crangon*, an experimental approach is recommended by exposing shrimps to different stress conditions, e.g. starvation or different temperatures.

4.6 Conclusive remarks

The objective of this thesis was to investigate biochemical properties that support adaptive physiological processes in *C. crangon* in a variable and changing environment. This extensive study contributed to a better understanding of the pronounced adaptive diversification of the biochemical structures and processes, reflected by comprehensive data of the midgut gland of *C. crangon*.

The high level of digestive enzyme polymorphism in *C. crangon* specimens seems to be an important feature for their biological success in the North Sea. This pronounced polymorphism has different impacts on the metabolism of North Sea shrimps. On the one hand, it affects the sequence of proteins necessary for the accumulation of lipids and it therefore also influences specific metabolic pathways (publication III). This change in the sequence of specific proteins is a challenge to the metabolism of *C. crangon*, because it most likely results in a limited lipid storage capacity (publication I). Lipid storage is important to overcome periods of food paucity and to fuel reproduction. However, in *C. crangon* the midgut gland does not function as principal energy depot. Instead, the midgut gland serves as a dynamic metabolic center that directly provides dietary energy necessary for diverse biological processes including reproduction (publication I).

This metabolic center seems to be supported by the expression of digestive enzyme isoforms, which improve the catabolic efficiency of *C. crangon* to obtain energy from different food sources (publication II). This dietary flexibility is an advantage to inhabit a variable environment, where food availability is frequently and seasonally modified.

On the other hand, the high mutation rate and high polymorphism level may also enhance the efficiency of metabolic processes in the midgut gland by producing enzymes with

different substrate affinities. For example, even if the main protease (trypsin) is not fully functional due to a mutation in its catalytic site (chapter 4), trypsin is substituted by several isoforms (publication II) of other enzymes that do the work, thus avoiding stress for *C. crangon*. This is supported by the fact that a large number of metabolite concentrations do not change, depending on the level of trypsin activities (chapter 4).

In this way, selection may favour digestive enzyme isoforms in *C. crangon*, because they increase the phenotypic robustness and provide a broader ecological tolerance, which is typical for species with large genomes (chapter 4). Apparently, pronounced polymorphism supports adaptive physiological processes in *C. crangon* allowing it to thrive in a variable and changing environment. Due to this flexibilty, *C. crangon* is probably well adapted to future global change scenarios.

4.7 Perspectives

This thesis is the product of an integrative research approach that included various disciplines. Here, I present the study of lipid storage capacities and dietary preferences via marker fatty acids, metabolic pathways, transcriptome, metabolites, and proteins to investigate the physiological performance of North Sea shrimps. While answering several important questions (chapters 1 to 4), the findings of this study have also triggered many new and exciting questions, which are summarized below and should be further investigated.

There are still a lot of knowledge gaps about the lipid metabolism in the midgut gland of Decapoda in general and brown shrimps specifically. Although we know now that *C. crangon* have limited lipid storage capacities (publication I), it is still necessary to find the reason behind this. In this thesis, two possible explanations for the limited lipid storage are proposed (publication III). The first one is the mutation in the binding site of the fatty acid-binding proteins, which serve as a lipid transport system within the cell. The second possible explanation presented here is the affected metabolic pathway in the synthesis of triacylglycerols, which are subsequently stored as a lipid reserve.

This study could only focus on a limited number of molecules involved in lipid metabolism, and therefore, a more comprehensive analysis of the entire transcriptome would be necessary. A comparison of the transcriptome of *C. crangon* with that of other decapod species with low and high lipid storage, respectively, would be of particular interest. Here, the shrimps *P. montagui* and *C. allmanni*, which have also been studied here, would make excellent

species for this comparison. This thesis reports on the lipid reserves in the midgut gland of these two species (publication I & chapter 4). Therefore, it would be interesting to find out, if the two *Crangon* species with their limited lipid storage capacities share metabolic properties. If so, it should be investigated to what extent they resemble *P. montagui,* a species with clearly higher lipid storage capabilities. Complementary to the transcriptome, a gene expression analysis could be a powerful tool for future assessments of the lipid metabolism and its regulation. Gene expression analysis could be a quantitative approach to provide evidence, how some biological processes (e.g. reproduction or feeding) modulate lipid metabolism in the brown shrimp. Finally, additional studies on e.g. hemolymph, ovaries, and eggs will be necessary to further understand the role of lipids in the reproduction of *C. crangon.*

This thesis greatly increased our understanding of the pronounced polymorphism that the enzymes of the midgut gland of *C. crangon* present. This fills major knowledge gaps and contributes to a better understanding of the physiological performance of brown shrimps in a variable environment. The level of enzyme polymorphism, previously reported as activity bands in the midgut gland of *C. crangon,* can also be observed in the transcripts of several enzymes. This polymorphism is variable and seems to be related to the functions of these digestive enzymes in the organism. Enzymes with a more specific role in metabolic pathways are usually more conservative in comparison to those that have diverse intracellular and extracellular functions. This study, therefore, proposes that the polymorphism of digestive enzymes in *C. crangon* represents an adaptive strategy that allows this species to inhabit an environment, where food quality and quantity change drastically between seasons (publication II).

Even if it is not possible to fully identify the source of this polymorphism, two likely possibilities were proposed: (I) the heterozygosity of genes coding for those proteins and, (II) alternative splicing events (chapter 4). However, a detailed assessment of the genome, in addition to the transcripts and their bioinformatic analysis, would be necessary to provide evidence of the source of the polymorphism. Alternatively, it would be possible to utilize new technologies such as PacBio sequencing, which has proven to be a powerful tool for the study of alternative splicing events.

Once the source of the polymorphism has successfully been determined - and if it is a combination of both processes as proposed above - it would be of utmost interest to develop this investigation further. Could alternative splicing be dynamically regulated in *C. crangon* by expressing isoforms with different affinities for the substrates and according to metabolic

needs, and subsequently, be a potent and metabolically inexpensive way to increase system robustness?

This study focussed on metabolites in the midgut gland of *C. crangon* and on differences between individuals with varying trypsin activities. No significant difference between these three groups of individuals was found (chapter 4). It was also possible to identify the different metabolites present in the midgut gland of the brown shrimp. Future studies should include an experimental approach, addressing questions regarding the modulation of metabolites under biological processes (e.g. developmental stage or reproduction), and external stressors (e.g. change of temperature, oxygen or food supply).

In the present study, the sequence of the serine proteinase trypsin is reported with a particular emphasis on the mutation of its catalytic triad (chapter 4), which has also been identified in other caridean shrimps. Following up on these results, the biochemical characterization of trypsin, together with the analysis of the enzyme chymotrypsin (another important serine proteinase) should be addressed in future studies. Since the data analyzed here are from a transcriptome source, it would be beneficial to complement them with an amino acid sequence analysis. It is therefore suggested to address the purification of these two enzymes, followed by mass spectrometry analysis, to determine the amino acid sequence. Ideally, this would be complemented by tertiary structure analysis, looking at substrate interactions and comparing those with non-mutated enzymes.

This research suggests that the high level of polymorphism in *C. crangon* most likely originates from its large genome and therefore has a high mutation rate with various molecular impacts on the metabolism. On the one hand, it may affect the capability to store lipids, but on the other hand, it may also help to make metabolic processes in the midgut gland more efficient by producing enzymes with different substrate affinities. This is an extraordinary example of how some molecular traits can have contrasting effects - advantages and disadvantages in an organism. These traits may allow the organism to cope with relevant environmental challenges.

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APPENDIX A

Quality assessment of the *C. crangon* midgut gland transcriptome assembly.

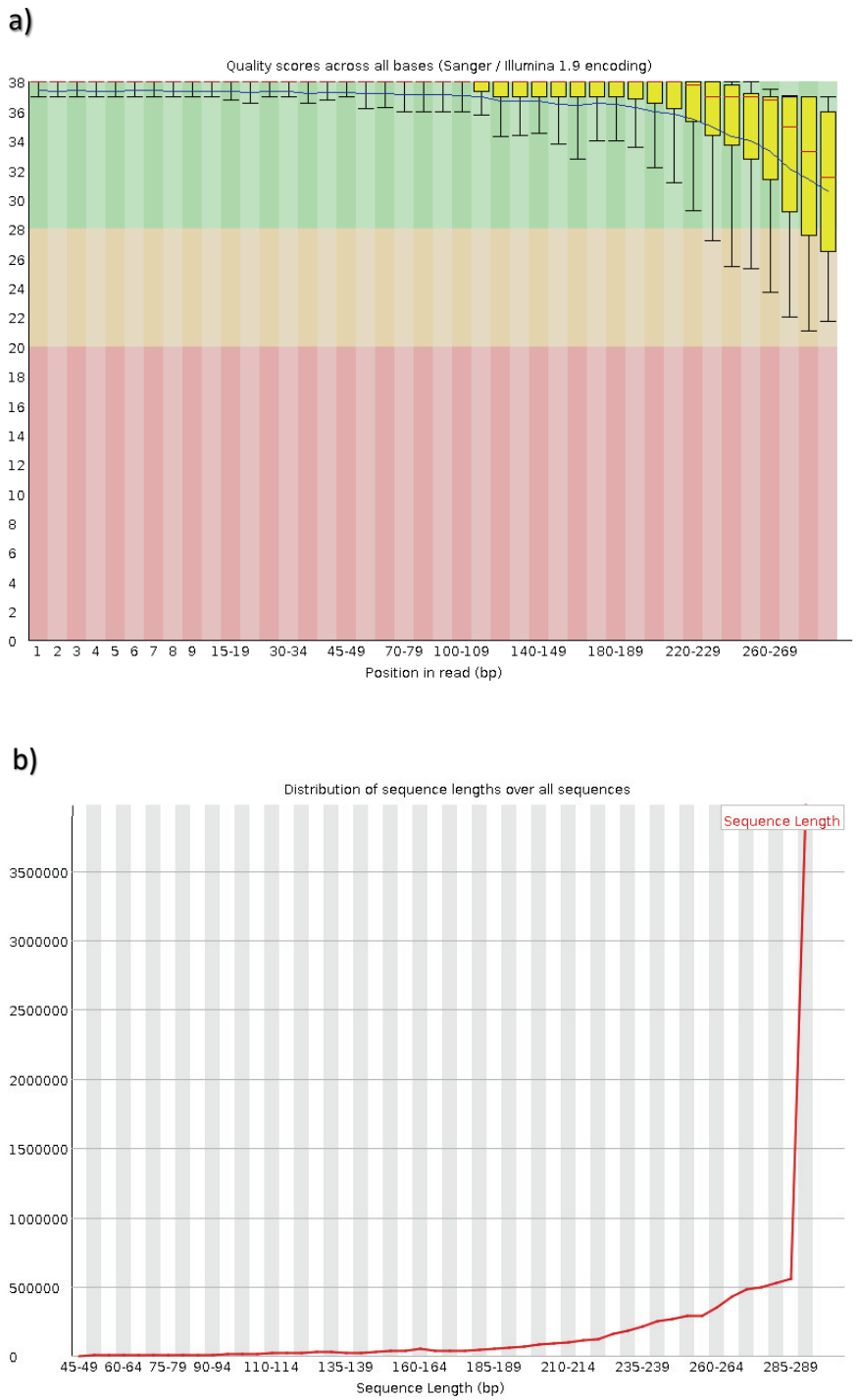


Fig. A1. Results of quality clipping a) per base sequence quality b) sequence length distribution.

APPENDIX B

de novo transcriptome assembly general information.

Table B1. Alignment statistics of *de novo* transcriptome assembly.

	No. of reads	Percentage
proper_pairs	26998122	71.14
improper_pairs	8337662	21.97
right_only	1851488	4.88
left_only	765915	2.02

Table B2. Assembly statatics of *de novo* transcriptome assembly.

	Assembly
No. of genes	56247
No. of transcripts	136016
N25	1862
N50	682
N75	346
Longest transcript	14702
Average transcript length	569
Median of transcript length	339
Shortest transcript	201
Total bases	77330318

TABLE B3. Full length transcript statistics.

	Percentage of protein length coverage	No. of transcripts
1	100	2295
2	90	805
3	80	582
4	70	474
5	60	458
6	50	531
7	40	625
8	30	809
9	20	876
10	10	391

APPENDIX C

General information about the functional annotation of *de novo* assembly.

Table C1. Funtional annotation statistics of *de novo* assembly.

	No. of hits
sprot_Top_BLASTX_hit	32619
TrEMBL_Top_BLASTX_hit	47149
RNAMMER	25
prot_id	42223
prot_coords	42223
sprot_Top_BLASTP_hit	24385
TrEMBL_Top_BLASTP_hit	30222
Pfam	23362
SignalP	2371
TmHMM	4712
eggnog	13489
gene_ontology_blast	31485
gene_ontology_pfam	15089
transcript	0
peptide	0

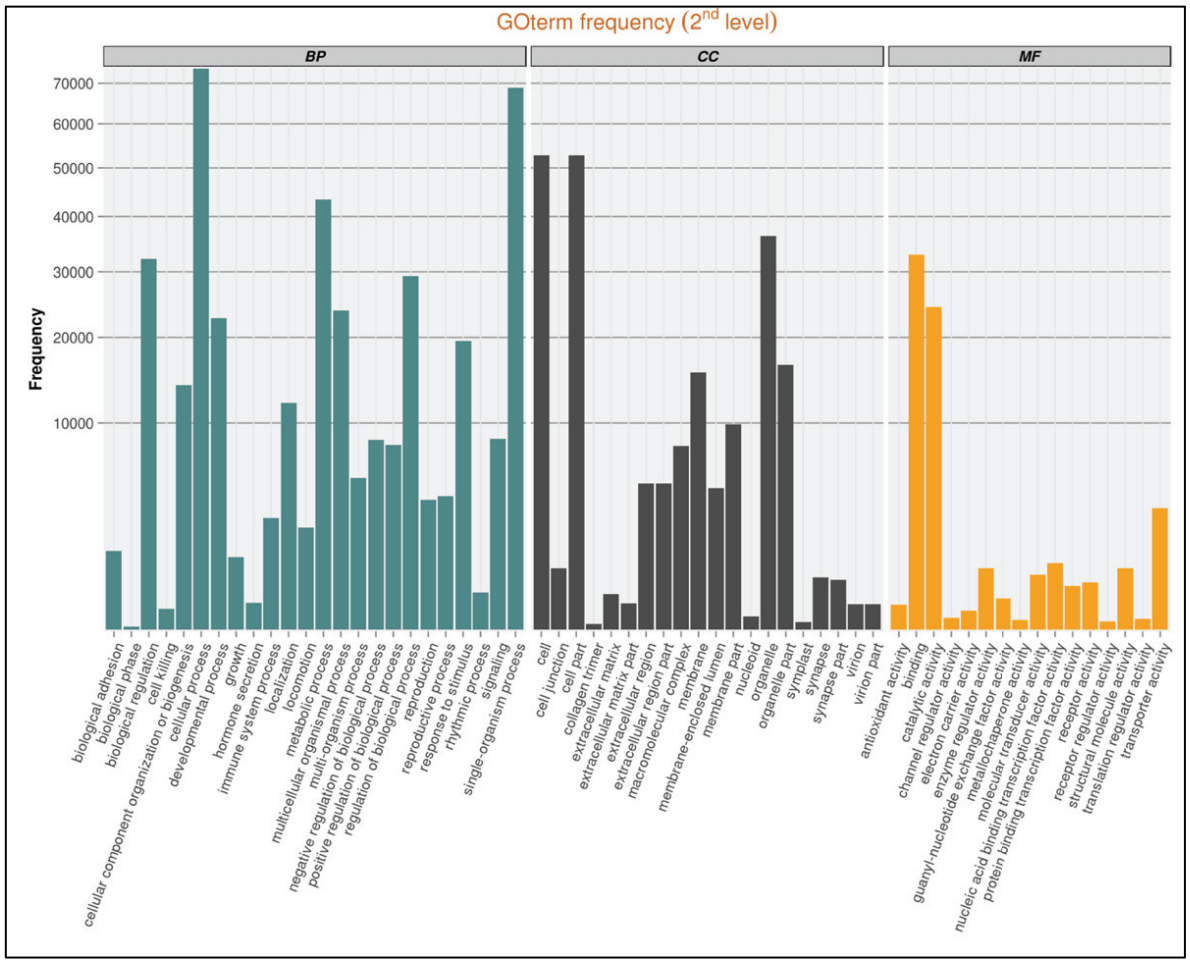
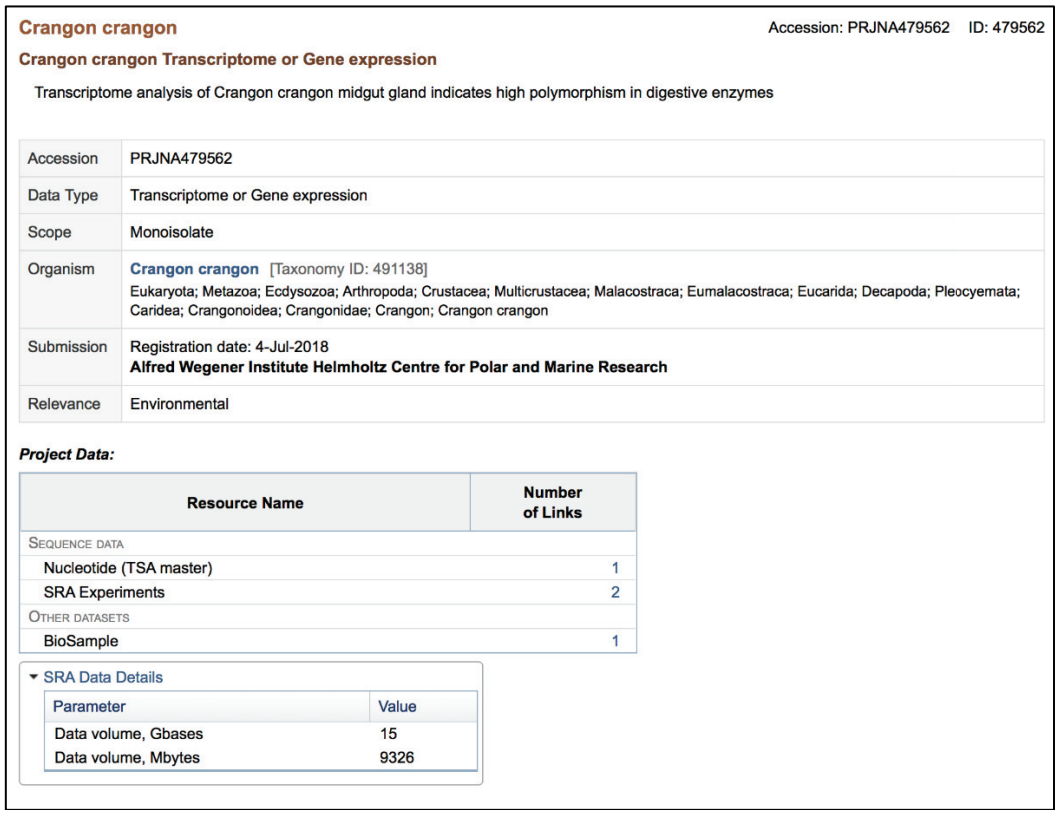


Fig. C1. Plot of GOterm frequency in trinotate report broken down to the 2nd level.

APPENDIX D

DATA PUBLISHED IN THE GenBank



Crangon crangon Accession: PRJNA479562 ID: 479562

Crangon crangon Transcriptome or Gene expression

Transcriptome analysis of Crangon crangon midgut gland indicates high polymorphism in digestive enzymes

Accession	PRJNA479562
Data Type	Transcriptome or Gene expression
Scope	Monoisolate
Organism	**Crangon crangon** [Taxonomy ID: 491138] Eukaryota; Metazoa; Ecdysozoa; Arthropoda; Crustacea; Multicrustacea; Malacostraca; Eumalacostraca; Eucarida; Decapoda; Pleocyemata; Caridea; Crangonoidea; Crangonidae; Crangon; Crangon crangon
Submission	Registration date: 4-Jul-2018 **Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research**
Relevance	Environmental

Project Data:

Resource Name	Number of Links
SEQUENCE DATA	
Nucleotide (TSA master)	1
SRA Experiments	2
OTHER DATASETS	
BioSample	1

SRA Data Details

Parameter	Value
Data volume, Gbases	15
Data volume, Mbytes	9326

Fig. D1. BioProject data.

Midgut gland transcriptome of Crangon crangon

Identifiers: BioSample: SAMN09580251; Sample name: Midgut gland transcriptome of Crangon crangon; SRA: SRS3506628

Organism: Crangon crangon
cellular organisms; Eukaryota; Opisthokonta; Metazoa; Eumetazoa; Bilateria; Protostomia; Ecdysozoa; Panarthropoda; Arthropoda; Mandibulata; Pancrustacea; Crustacea; Multicrustacea; Malacostraca; Eumalacostraca; Eucarida; Decapoda; Pleocyemata; Caridea; Crangonoidea; Crangonidae; Crangon

Package: Invertebrate; version 1.0

Attributes:

isolate	not applicable
host	not applicable
collection date	26-Jun-2015
geographic location	North Sea: Germany
tissue	Midgut gland
collected by	Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research
development stage	Adults
latitude and longitude	53.48 N 8.10 E
sex	pooled male and female

Description: Transcriptome analysis of Crangon crangon midgut gland indicates high polymorphism in digestive enzymes

BioProject: PRJNA479562 Crangon crangon
Retrieve all samples from this project

Submission: Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, Diana Martinez Alarcon; 2018-07-04

Accession: SAMN09580251 ID: 9580251
BioProject SRA Nucleotide

Fig. D2. BioSample data.

SRX4346762: RNA-Seq of Crangon crangon: adult pooled sample midgut gland: high trypsin activity
1 ILLUMINA (Illumina MiSeq) run: 11.3M spots, 6.7G bases, 3.9Gb downloads

Design: mRNA sequencing of Crangon crangon midgut gland (adult) for transcriptome assembly (high trypsin activity)

Submitted by: Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research

Study: Crangon crangon Transcriptome or Gene expression
PRJNA479562 • SRP152406 • All experiments • All runs
show Abstract

Sample: Midgut gland transcriptome of Crangon crangon
SAMN09580251 • SRS3506628 • All experiments • All runs
Organism: Crangon crangon

Library:
Name: A_S1
Instrument: Illumina MiSeq
Strategy: RNA-Seq
Source: TRANSCRIPTOMIC
Selection: RANDOM
Layout: PAIRED

Runs: 1 run, 11.3M spots, 6.7G bases, 3.9Gb

Run	# of Spots	# of Bases	Size	Published
SRR7477266	11,322,410	6.7G	3.9Gb	2018-07-06

Fig. D3. SRA of samples with high trypsin activity.

SRX4346761: RNA-Seq of Crangon crangon: adult pooled sample midgut gland: low trypsin activity
1 ILLUMINA (Illumina MiSeq) run: 13.5M spots, 8.1G bases, 4.8Gb downloads

Design: mRNA sequencing of Crangon crangon midgut gland (adult) for transcriptome assembly (low trypsin activity)

Submitted by: Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research

Study: Crangon crangon Transcriptome or Gene expression
PRJNA479562 • SRP152406 • All experiments • All runs
show Abstract

Sample: Midgut gland transcriptome of Crangon crangon
SAMN09580251 • SRS3506628 • All experiments • All runs
Organism: Crangon crangon

Library:
Name: B_S2
Instrument: Illumina MiSeq
Strategy: RNA-Seq
Source: TRANSCRIPTOMIC
Selection: RANDOM
Layout: PAIRED

Runs: 1 run, 13.5M spots, 8.1G bases, 4.8Gb

Run	# of Spots	# of Bases	Size	Published
SRR7477267	13,537,417	8.1G	4.8Gb	2018-07-06

Fig. D4. SRA of samples with low trypsin activity.

Table D1. Asseccion number asigned by the GenBank database to the submitted sequences.

ENZYME	EC ID	ACCESSION NUMBER									
CATHEPSIN L	EC 3.4.22.15	MH069296	MH069316	MH069336	MH069356	MH069376	MH069396	MH069416	MH069436	MH069456	MH069476
		MH069297	MH069317	MH069337	MH069357	MH069377	MH069397	MH069417	MH069437	MH069457	MH069477
		MH069298	MH069318	MH069338	MH069358	MH069378	MH069398	MH069418	MH069438	MH069458	MH069478
		MH069299	MH069319	MH069339	MH069359	MH069379	MH069399	MH069419	MH069439	MH069459	MH069479
		MH069300	MH069320	MH069340	MH069360	MH069380	MH069400	MH069420	MH069440	MH069460	MH069480
		MH069301	MH069321	MH069341	MH069361	MH069381	MH069401	MH069421	MH069441	MH069461	MH069481
		MH069302	MH069322	MH069342	MH069362	MH069382	MH069402	MH069422	MH069442	MH069462	MH069482
		MH069303	MH069323	MH069343	MH069363	MH069383	MH069403	MH069423	MH069443	MH069463	MH069483
		MH069304	MH069324	MH069344	MH069364	MH069384	MH069404	MH069424	MH069444	MH069464	MH069484
		MH069305	MH069325	MH069345	MH069365	MH069385	MH069405	MH069425	MH069445	MH069465	MH069485
		MH069306	MH069326	MH069346	MH069366	MH069386	MH069406	MH069426	MH069446	MH069466	MH069486
		MH069307	MH069327	MH069347	MH069367	MH069387	MH069407	MH069427	MH069447	MH069467	MH069487
		MH069308	MH069328	MH069348	MH069368	MH069388	MH069408	MH069428	MH069448	MH069468	MH069488
		MH069309	MH069329	MH069349	MH069369	MH069389	MH069409	MH069429	MH069449	MH069469	MH069489
		MH069310	MH069330	MH069350	MH069370	MH069390	MH069410	MH069430	MH069450	MH069470	MH069490
		MH069311	MH069331	MH069351	MH069371	MH069391	MH069411	MH069431	MH069451	MH069471	MH069491
		MH069312	MH069332	MH069352	MH069372	MH069392	MH069412	MH069432	MH069452	MH069472	MH069492
		MH069313	MH069333	MH069353	MH069373	MH069393	MH069413	MH069433	MH069453	MH069473	MH069493
		MH069314	MH069334	MH069354	MH069374	MH069394	MH069414	MH069434	MH069454	MH069474	MH069494
		MH069315	MH069335	MH069355	MH069375	MH069395	MH069415	MH069435	MH069455	MH069475	
CHITINASE	EC 3.2.1.14	MH069216	MH069227	MH069238	MH069249	MH069260	MH069271	MH069282	MH069293		
		MH069217	MH069228	MH069239	MH069250	MH069261	MH069272	MH069283	MH069294		
		MH069218	MH069229	MH069240	MH069251	MH069262	MH069273	MH069284	MH069295		
		MH069219	MH069230	MH069241	MH069252	MH069263	MH069274	MH069285			
		MH069220	MH069231	MH069242	MH069253	MH069264	MH069275	MH069286			
		MH069221	MH069232	MH069243	MH069254	MH069265	MH069276	MH069287			
		MH069222	MH069233	MH069244	MH069255	MH069266	MH069277	MH069288			
		MH069223	MH069234	MH069245	MH069256	MH069267	MH069278	MH069289			
		MH069224	MH069235	MH069246	MH069257	MH069268	MH069279	MH069290			
		MH069225	MH069236	MH069247	MH069258	MH069269	MH069280	MH069291			
		MH069226	MH069237	MH069248	MH069259	MH069270	MH069281	MH069292			
PHOSPHOLIPASE A_2	EC 3.1.1.4	MH055777	MH055782	MH055787	MH055792						
		MH055778	MH055783	MH055788	MH055793						
		MH055779	MH055784	MH055789	MH055794						
		MH055780	MH055785	MH055790	MH055795						
		MH055781	MH055786	MH055791	MH055796						
TRIACYLGLYCEROL LIPASE	EC 3.1.1.3	MH055763	MH055768	MH055773							
		MH055764	MH055769	MH055774							
		MH055765	MH055770	MH055775							
		MH055766	MH055771	MH055776							
		MH055767	MH055772								
ALPHA AMYLASE	EC 3.2.1.1	MH055751	MH055756	MH055761							
		MH055752	MH055757	MH055762							
		MH055753	MH055758								
		MH055754	MH055759								
		MH055755	MH055760								
TRYPSIN	EC 3.4.21.4	MH035874	MH035879								
		MH035875	MH035880								
		MH035876	MH035881								
		MH035877	MH035882								
		MH035878	MH069215								

INTERNATIONAL CONFERENCE PARTICIPATION

July 2019. "Molecular adaptive mechanisms of the brown shrimp *Crangon crangon* to survive in a variable environment" at the "Annual meeting of The Society for Experimental Biology". Seville, Spain.

May 2018. "Hepatopancreas transcriptome analysis of the brown shrimp, *Crangon crangon*, reveals expression of polymorphic enzymes" at the "International Crustacean Congress-IX". Washington D.C., USA. (Student presentation award).

September 2017. "Is the heterogeneity of digestive enzymes in the North Sea shrimp *Crangon crangon* (Decapoda) an adaptation to a variable environment?" in "Physiomar 17". Cripps Court, Magdalene College, Cambridge, UK. (Student presentation award).

March 2017. "Integrated gene expression and protein analyses of digestive enzymes in the brown shrimp *Crangon crangon*" in "Crustaceologen Tagung". Berlin, Germany.

February 2017. "Transcriptome and gene expression analysis of the brown shrimp *Crangon crangon* reveals seasonal modulation of digestive enzymes" in "ASLO 2017, From the Mountaind to the Sea". Hawaii, USA.

September 2016. "Lipid metabolism of the North Sea shrimps" in "YOUMARES 7 Young Marine Researchers". Hamburg, Germany.

February 2016. "Ecophysiological performance and life cycle strategies of North Sea shrimps" in "Shaping the picture: getting relevant information from transcriptomic data sets". Carolinensiel, Germany.

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ERKLÄRUNG

Hiermit erkläre ich, dass ich die Doktorarbeit mit dem Titel :

"Ecophysiological performance and life cycle strategies of North Sea shrimps"

selbstständig verfasst und geschrieben habe und außer den angegebenen Quellen keine weiteren Hilfsmittel verwendet habe.

Ebenfalls erkläre ich hiermit, dass es sich bei den von mir abgegebenen Arbeiten um drei identische Exemplare handelt.

Bremen, den 25.09.2019

Diana Martinez Alarcon