

Assessing the Presence of Chitinases in the Digestive Tract and their Relationship to Diet and Morphology in Freshwater Fish

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LIST OF ABBREVIATIONS

| | |
|-----------------|---|
| AMCase | Acidic mammalian chitinase |
| AFCase-1 | Acidic fish chitinase-1 |
| AFCase-2 | Acidic fish chitinase-2 |
| BM | Body mass |
| Chia | Acidic chitinase |
| ChT | Chitotriosidase |
| chit1 | Chitotriosidase |
| DI-MS | Direct Infusion – Mass Spectrometry |
| Fcase-3 | Fish chitinase-3 |
| fChi1 | Flounder chitinase 1 |
| fChi2 | Flounder chitinase 2 |
| fChi3 | Flounder chitinase 3 |
| GlcNac | N-acetyl-D-glucosamine |
| GH | Glycosylhydrolases |
| GM | Gut mass |
| IDP-PACU | Institución Pública Desconcentrada de Pesca y Acuicultura |
| IL | Intestinal length |
| NAGase | β -(1,4) N-acetylglucosaminidases |
| PCA | Principal Component Analysis |
| PLS-DA | Partial Least Squares Discriminant Analysis |
| SL | Standard length |
| tGCase | Toad gastric chitinase |
| tPCase | Toad pancreatic chitinase |
| RGL | Relative gut length |
| RGM | Relative gut mass |
| SCFA | Short-chain fatty acids |
| SIA | Stable Isotope Analysis |
| ZI | Zihler's index |

SUMMARY

Increased efforts are needed to increase the output of all food-producing sectors to keep up with the surging demand of the growing world population, especially in developing countries. Aquaculture products are generally a cheap commodity and thus often the sole available option of animal protein for the world's poor. However, intensifying aquaculture is not without its drawbacks. Paradoxically, increasing the production of harvested fish has led to overfishing, due to the use of fish meal in most aquaculture systems. Alternative unutilised resources could be used instead of fish meal, but sometimes contain considerable amounts, of possibly indigestible, chitin, which is resilient to most physicochemical processes, but hydrolysed by an enzyme.

According to the available current knowledge, some fish have high concentrations of chitinases in the intestinal tract. Most of the enzymatic activity in the gastrointestinal tract is produced by the fish itself. It is possible that fish are able to digest chitin as a source of energy and nutrients. Alternatively, fish chitinases' primary function is the chemical disruption of the exoskeleton or other chitinous outer structures of prey, so that the inner nutrients can be reached by enzymes or to prevent intestinal blockage by these structures. Additionally, in past research, several possible relationships between chitinase-activity and morphology/diet have been investigated; however, results are inconclusive.

In this work, it was attempted to establish whether the examined fish species are able to digest chitin and whether any correlation with diet or morphology could be found.

Metabolomics was performed on the fish species to determine the number of chitin-metabolites in blood and gastrointestinal tract. Stable isotope analysis was performed to measure diet and trophic level positioning; however, results were not analysed in time. Dissections were performed to obtain morphometric data and gross morphology of the gastrointestinal tract.

Our findings indicate that all examined freshwater fish are able to digest chitin to a certain extent and that the chitin-metabolites are absorbed. A correlation between gastrointestinal length and dietary preference was found. However, a relationship between chitin-intake/gastrointestinal length and chitinase-activity could not be established. Our results do indicate a relationship between the amount of mechanical processing and chitinase-activity, which might imply a role as a chemical disruptor of chitin.

SAMENVATTING

Meer inspanningen zijn nodig om de productie van alle voedselproducerende sectoren te vergroten om de stijgende vraag van de groeiende wereldbevolking, die voornamelijk in ontwikkelingslanden plaatsvindt, bij te houden. Aquacultuurproducten zijn goedkoop en aldus vaak de enige beschikbare optie van dierlijke eiwitten voor de armen in de wereld. De intensivering van de aquacultuur is echter niet zonder nadelen. Paradoxaal genoeg heeft het verhogen van de productie van geogoste vis geleid tot overbevissing, vanwege het gebruik van vismeel in de meeste aquacultuursystemen. Alternatieve ongebruikte bronnen kunnen worden gebruikt in plaats van vismeel, maar bevatten soms aanzienlijke hoeveelheden, mogelijk onverteerbaar, chitine, dat resistent is tegen de meeste fysisochemische processen, maar gehydrolyseerd kan worden door een enzym.

Volgens de beschikbare huidige kennis hebben sommige vissen hoge concentraties chitinasen in het darmkanaal. De enzymatische activiteit in het maagdarmkanaal wordt grotendeels door de vis geproduceerd. Het is mogelijk dat vissen chitine kunnen verteren als een bron van energie en voedingsstoffen. Een andere mogelijkheid is dat de primaire role van chitinasen in vissen beperkt is tot het chemisch verstoren van het exoskelet of andere chitine-bevattende structuren afkomstig van een prooi, zodat de beter verteerbare nutriënten in contact kunnen komen met de andere enzymen, oftewel wordt hiermee mogelijke obstructie van het maagdarmkanaal door deze structuren vermeden. Bovendien zijn in eerder onderzoek verschillende mogelijke verbanden tussen chitinase-activiteit en morfologie / dieet onderzocht; de resultaten zijn echter niet doorslaggevend.

In dit werk werd geprobeerd vast te stellen of de onderzochte vissoorten in staat zijn om chitine te verteren en of er een verband met dieet of morfologie kon worden aangetoond.

Metabolomics werd uitgevoerd op de vissoorten om de hoeveelheid chitine-metabolieten in het bloed en het maagdarmkanaal te bepalen. Stabiele isotopenanalyse werd uitgevoerd om het dieet en de positionering van het trofische niveau te meten; de resultaten hiervan werden echter niet op tijd geanalyseerd. Dissecties werden uitgevoerd om morfometrische gegevens en morfologie van het maagdarmkanaal te verkrijgen.

Onze bevindingen geven aan dat alle onderzochte zoetwatervissen chitine tot op een zeker niveau kunnen verteren en dat de chitine-metabolieten worden geabsorbeerd. Een correlatie tussen gastro-intestinale lengte en dieetvoorkeur werd gevonden. Een relatie tussen chitine-inname / gastro-intestinale lengte en chitinase-activiteit kon echter niet worden vastgesteld. Onze resultaten wijzen op een verband tussen de hoeveelheid mechanische verwerking en chitinase-activiteit, welke kan wijzen op hun rol in het chemisch verstoren van chitine.

1. LITERATURE REVIEW

1.1. INTRODUCTION

The world population and its need for food are rapidly increasing; according to projections¹, it will exceed the nine billion mark by 2038. The highest rising rates of growth are projected to be in developing countries, where the standard of living for many inhabitants is low. Simultaneously, it has been projected¹ that the per capita rates of animal protein consumption will rise; thus, there is a need for increased efforts in livestock production (Tilman et al., 2011). On a global scale, aquaculture has a critical contribution toward feeding the population (Chamberlain and Rosenthal, 1995). In developing areas, fish is often the cheapest and thus only available animal protein for the poor (Finegold, 2009). In the past few decades, aquaculture has been one of the fastest-growing food-producing sectors, increasing from 12 million metric tons in 1985 to 45 million metric tons in 2004 (Diana, 2009). However, this enormous growth is not without its drawbacks. In some areas, aquaculture has caused damage to aquatic life and biodiversity by destroying habitats, waste disposal, pathogen invasion, and the introduction of exotic species (Naylor et al., 2000). Additionally, it appears that despite its remarkable growth, aquaculture production will not be able to keep up with the growing demand of the world population, unless significant changes to increase sustainability and regulation are made (Chamberlain and Rosenthal, 1995; FAO, 2018). This especially endangers the employment and food security of people living in developing countries, where 92.3% of the global culture harvest takes place (Diana, 2009).

One of the major problems faced by aquaculture is its dependence on fish meal and fish oil from wild pelagic fish to formulate feed for the harvested fish (Olsen and Hasan, 2012). This may lead to over-fishing of an aquatic resource, which could be directly used as food for humans (Tacon and Metian, 2009). However, there are several more sustainable alternatives for fish meal, though more studies are required to measure their efficiency in practice. Alternatives include seafood by-products (e.g. crab shell waste) or use of krill, which is the largest underutilised ocean resource (Naylor et al., 2009). Another alternative venture is the use of insects, which have been reared on waste products, as fish feed (van Huis and Oonincx, 2017). The commonality between these alternatives is that they have considerable chitin content.

Chitin the most abundant carbohydrate of the marine environment and is thus likely to be the most frequently encountered carbohydrate by fish (Barikani et al., 2014). However, the extent to which it is digested by fish and their importance in captive fish nutrition has been a source of debate over the last few decades. Several studies have investigated the inclusion of chitin in the diet of cultured fish, and a plethora of studies have focused on replacement diets with insects or crustaceans (Henry et al., 2015). However, the results of these studies are contradictory; some have produced positive results of chitin or material containing chitin supplementation in a particular species, whereas different research has produced a negative effect of supplementation on the same species. More often than not, when negative results are produced, the authors propose that these effects are caused by chitin. It is often thought that chitin is indigestible by fish, but such a claim is not founded on any actual data (Rust, 2002).

Chitin is very resistant to chemical degradation, but a sequence of enzymes, chitinase and NAGase, is able to hydrolyse chitin into monomers of N-acetyl-glucosamine (Dahiya et al., 2006). It has been reported that in several vertebrates and especially fish, chitinase activity is present in the gastrointestinal system (Jeuniaux, 1993). However, several aspects of fish chitinase remain controversial. Although fish chitinase genes have been isolated in several species, there is no consensus on whether the endogenous or exogenous source (i.e. bacterial or prey chitinases) are the most important contributor of chitinolytic activity (Goodrich and Morita, 1977a; Lindsay and Gooday, 1985; Ikeda et al., 2017a). Their exact function remains unclear, though mostly a role in either immunity, digestion, or a combination of both has been proposed (Gutowska et al., 2004; Zhang et al., 2012).

¹ <http://databank.worldbank.org/data/source/population-estimates-and-projections> (Last consulted on 14/02/2019)

Although several links between the presence of chitinase and the fishes' diet and gastrointestinal morphology have been proposed, the currently available data is inconclusive (Lindsay, 1984; Gutowska et al., 2004; German et al., 2015).

More research is needed to know to what extent, if any, chitin is digested and utilisable by fish. Additionally, if a link can be established between chitin digestibility and diet or gastrointestinal morphology, these could be used as predictors to which fish species are likely to utilise chitin. Future endeavours should be focused on these suitable candidates for an alternative chitin-containing meal diet to reduce the currently unsustainable practice of feeding fish meal in aquaculture systems.

1.2. CHITIN

1.2.1. 2D-structure

Chitin ($C_8H_{13}O_5N$)_n, a naturally occurring mucopolysaccharide, is the second most abundant biopolymer produced annually by biosynthesis in the world after cellulose (Roberts, 1992; Cohen-Kupiec and Chet, 1998; Barikani et al., 2014). The idealised structure of chitin is similar to that of cellulose as shown in *Figure 1A*, the key difference being that in chitin the C2-hydroxyl group has been substituted with an acetamido-group (Roberts, 1992; Ravi Kumar, 2000; Zargar et al., 2015). Both are linear polymers consisting of repeating monosaccharides; cellulose consists of β-(1→4) linked D-glucose units, whereas chitin is a poly[β-(1→4)-N-acetyl-D-glucosamine] (GlcNac) (Hamid et al., 2013; Zargar et al., 2015).

According to Roberts (1992) and Zargar et al. (2015), chitin is rarely found in nature in its pure ideal state; rather, it often complexes with polysaccharides, proteins and minerals. Even after stringent isolation and purification, the obtained polymer chains will have varying levels of acetylation. Thus, in nature, chitin should be considered as a copolymer made out of two distinct monomer building blocks; in chitin the proportion of GlcNac residues is higher than that of the D-glucosamine (GlcN), whereas the reverse is the case in chitosan [*Figure 1B*] (Roberts, 1992; Zargar et al., 2015).

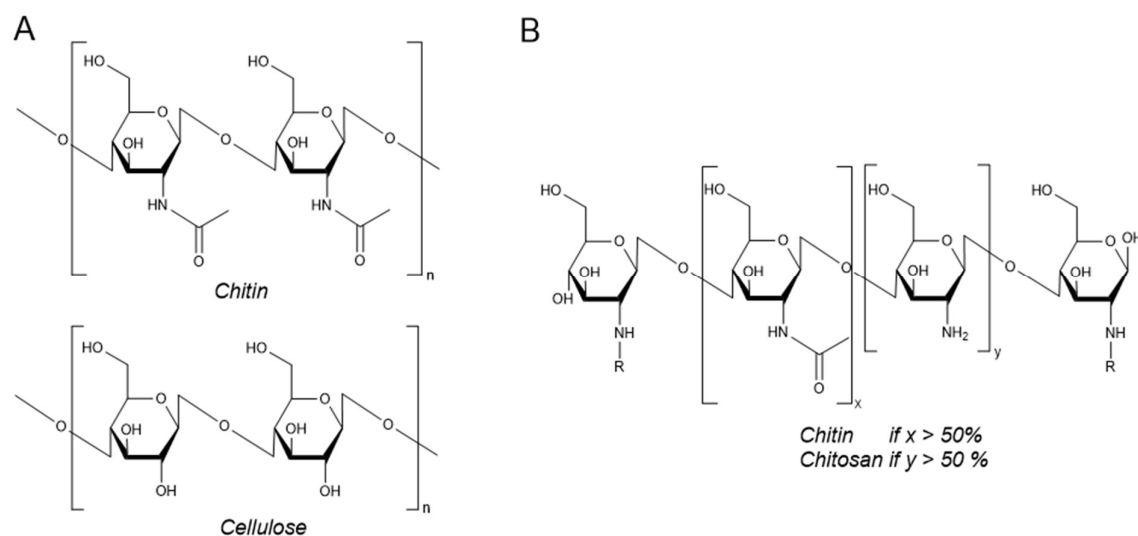


Figure 1: A. Haworth projections of the idealised structure of chitin and cellulose. B. Haworth projection of natural occurring chitin/chitosan copolymer

1.2.2. 3D-structure and crystallography

According to X-ray diffraction studies, chitin has a highly organised crystalline structure and has been found in three distinct polymorphic forms in nature: α-, β-, and γ -chitin, the difference between these forms lies in the packing and polarities of the adjacent chains in successive sheets (Rudall, 1963; Minke and Blackwell, 1978; Roberts, 1992; Muzzarelli, 2011).

A review by Zargar et al. (2015) describes all types of chitin as being comprised of stacks of chains that are joined together by the C=O...NH bonds found in the N-acetyl group of each GlcNac-monomer, in each stack the chain direction remains the same. How these stacks come together in a crystalline structure will determine the polymorphic form of chitin [Figure 2] (Zargar et al., 2015).

The neighbouring stacks of α -chitin consistently have opposing chain directions; in other words, the adjacent chains are arranged in an anti-parallel fashion. Conversely, in β -chitin, the adjacent chains are parallel to each other. Lastly, in the γ -conformation, the third chain has an opposing direction to the previous two neighbouring unidirectional chains [Figure 2] (Zargar et al., 2015). In the past some authors have suggested that the γ -polymorph is a mixture of α - and β -chitin, however, it has been recently proven that the third confirmation is distinct from the other two (Roberts, 1992; Jang et al., 2004; Kaya et al., 2017).

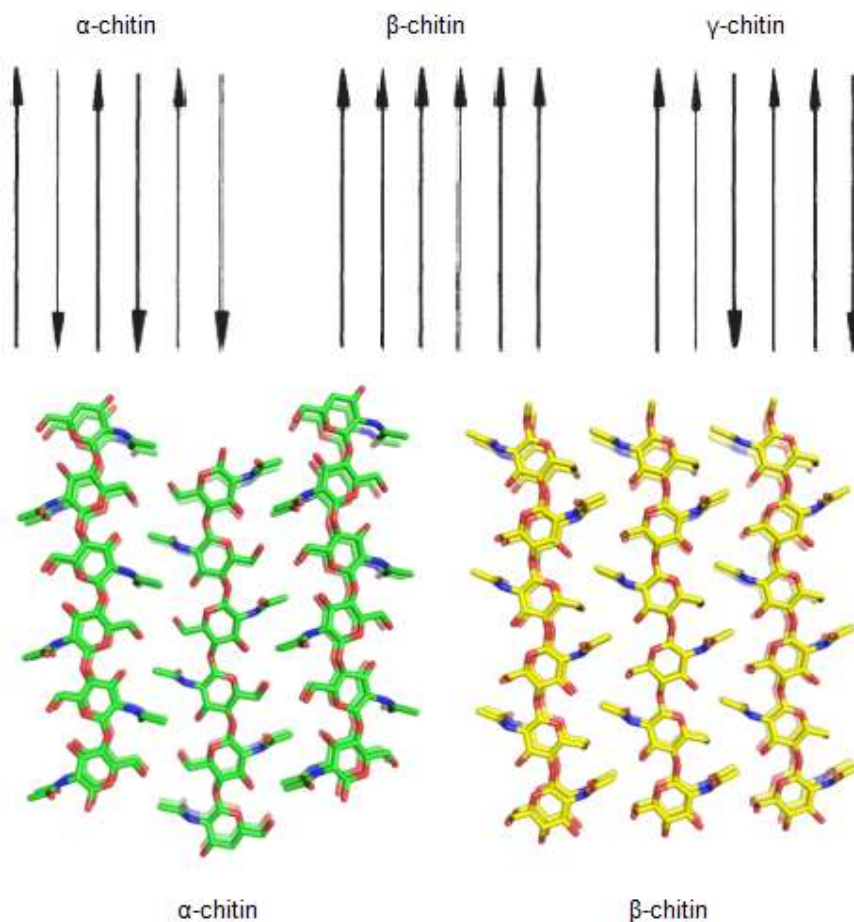


Figure 2: Up: Diagrammatic representation of the chitin chain arrangement of the three polymorphic forms (α -, β -, and γ - chitin) viewed in the plane of the sugar rings. Adapted from Roberts (1992). Down: Structures of α - and β -chitin, the chain direction in the stacks (shown here is two stacked polymers) is the same, whereas the direction of the chains in the adjacent stacks is either opposite (α - chitin) or the same (β -chitin). Adapted from Hurlburt et al. (2018).

1.2.3. Chemical properties

Chitin is highly hydrophobic and insoluble water and most organic solvents, it owes these properties to the presence of the acetyl amine group which generates inter- and intramolecular hydrogen bonds which make the chitin highly aggregated (Ravi Kumar, 2000; Kumirska et al., 2011; Zargar et al., 2015; Salaün et al., 2017). It is soluble in solvents such as isopropanol, hexafluoroacetone and hexafluoroisopropyl alcohol. Furthermore, hydrolysis resulting in relatively pure D-glucosamine is possible with concentrated acids under rigorous conditions (Ravi Kumar, 2000; Salaün et al., 2017).

The solubility of chitin is mainly dependent on the degree of N-acetylation as such chitosan, more than 50 % deacetylated chitin, is soluble in dilute aqueous acidic solutions such as acetic acid and formic acid (Ravi Kumar, 2000; Zargar et al., 2015).

Out of the three polymorphs, α -chitin is the most stable allomorph due to additional inter-chain hydrogen bonding which is absent in the other conformations, making it less susceptible to acid hydrolysis and intracrystalline swelling (Muzzarelli, 2011; Salaün et al., 2017). In nature, chitin is biologically degraded by specific enzymes referred to as chitinases or non-specific enzymes such as lysozyme (Hamid et al., 2013; Salaün et al., 2017).

1.2.4. Function and distribution

Chitin's role is that of a structural polymer similar to cellulose in plants and collagen in the higher animals (Roberts, 1992; Zargar et al., 2015). It is predominantly found as organised crystalline microfibrils forming key structural components in the exoskeleton of arthropods or the cuticle of molluscs, annelids (Rudall and Kenchington, 1973; Roberts, 1992; Zargar et al., 2015). Chitin is especially abundant in the marine environment, where it is estimated that the annual biosynthesis ranges from 10^{10} to 10^{11} metric tons (Gooday, 1990).

It was long believed that vertebrates did not contain any chitin, it was suggested that the enzyme responsible for chitin synthesis, a chitin synthase, was lost somewhere along with the evolutionary split of protostomes and deuterostomes, with the former retaining the chitin synthase (Rudall and Kenchington, 1973). However, a recent study has proven this to be untrue by isolating chitin synthase and chitin from several fishes and amphibian species (Tang et al., 2015).

Out of the three conformations, the α -allomorph is the most abundant; appearing in crustacean exoskeletons, insects' cuticles, and fungal cell walls, among others (Roberts, 1992; Muzzarelli, 2011). The less common β -chitin has been found in squid pens, pogonophoran tubes, and so on (Rudall, 1963; Muzzarelli, 2011). The third and least common conformation, γ -chitin, has been found in the cocoon of several arthropod species (Zargar et al., 2015; Kaya et al., 2017).

It has been suggested that the different polymorphs are related to variations in function; α -chitin has been mostly found in structures where hardness is required, whereas, β - and γ -chitin are commonly found in robust yet flexible structures (Jang et al., 2004; Kaya et al., 2017). As the three allomorphs have been isolated from the same organism, a relation to function seems more likely than a link to phylogenetic grouping (Roberts, 1992).

1.3. CHITINASE

1.3.1. Nomenclature and classification

In the current literature, there are some inconsistencies regarding the usage of names for enzymes that are involved in the lysis of chitin; therefore, it is essential to clarify specific terms. In a broad sense chitinases or chitinolytic enzymes, part of the glycosylhydrolase family, are defined as enzymes which catalyse the random hydrolysis of β -1,4-glycoside bonds between GlcNac-units of chitin and chitooligomers (Muzzarelli, 1993; Stoykov et al., 2015). If the mechanism for chitin degradation is not specified, the more general term 'chitinoclastic' is used (Gooday, 1994). There is also a group of chitinase-like lectins which are able to bind chitin; however, these proteins completely lack any chitinolytic activity due to substitutions in their key catalytic residues making them unable to donate a proton required for hydrolysis (Bussink et al., 2007; Funkhouser and Aronson, 2007).

Most commonly the chitinases have been divided into two major categories based on their functional characteristics: endochitinases and exo-chitinases, the latter is even further subdivided into chitobiosidase and β -(1,4) N-acetyl glucosaminidases (Cohen-Kupiec and Chet, 1998; Dahiya et al., 2006; Hamid et al., 2013). The endochitinase (E.C.3.2.1.14), also referred to as chitinase² (in a strict sense), cleaves chitin at random internal β -1,4-glycoside bonds and thus generating the dimer diacetylchitobiose and low molecular mass multimers such as chitotriose and chitotetraose (Dahiya et al., 2006; Hamid et al., 2013).

The chitobiosidases (EC 3.2.1.30) catalyse the progressive release of diacetylchitobiose starting at the non-reducing end of chitin (Cohen-Kupiec and Chet, 1998; Hamid et al., 2013). Lastly, the β -(1,4) N-acetyl glucosaminidases (NAGase) (E.C. 3.2.1.29) cleave the end-products of endochitinases and chitobiosidases, resulting in monomers of GlcNac (Cohen-Kupiec and Chet, 1998; Dahiya et al., 2006). This enzymatic hydrolysis through the combined action of endo-and exochitinases of chitin to N-acetylglucosamine units is often referred to as the chitinolytic system (Jeuniaux, 1993). An overview of these enzymes, their substrate and end-products are illustrated in Figure 3.

The bacterial lysozyme (E.C. 3.2.1.17), also referred to as N-acetylmuramylhydrolase, has a broad substrate specificity and also shows some chitinolytic activity towards chitin and chito-oligomers though weaker than that of endochitinase³ (Fänge et al., 1979; Clark et al., 1988). Similar to endochitinase, lysozyme shows no activity towards diacetylchitobiose and is thus unable to achieve complete degradation of chitin (Powning and Irzykiewicz, 1966; Yano, 1996).

The Enzyme Nomenclature Committee of the International Union of Biochemistry and Molecular Biology has merged the chitobiosidases and N-acetyl glucosaminidases with another group of enzymes, capable of hydrolysing N-acetylglucosides and N-acetylgalactosides from the non-reducing end, into a group named the β -N-acetylhexosaminidases⁴ (E.C. 3.2.1.52) (Patil et al., 2000). However, this thesis will use the former nomenclature to make a distinction between the three types of enzymes due to their differing specificity as well as their varying affinity which is mostly dependent on the chain length of the substrate (Patil et al., 2000).

² <https://enzyme.expasy.org/EC/3.2.1.14> (Last consulted on 20/02/2019)

³ <https://enzyme.expasy.org/EC/3.2.1.17> (Last consulted 20/02/2019)

⁴ <https://enzyme.expasy.org/EC/3.2.1.52> (Last consulted 21/02/2019)

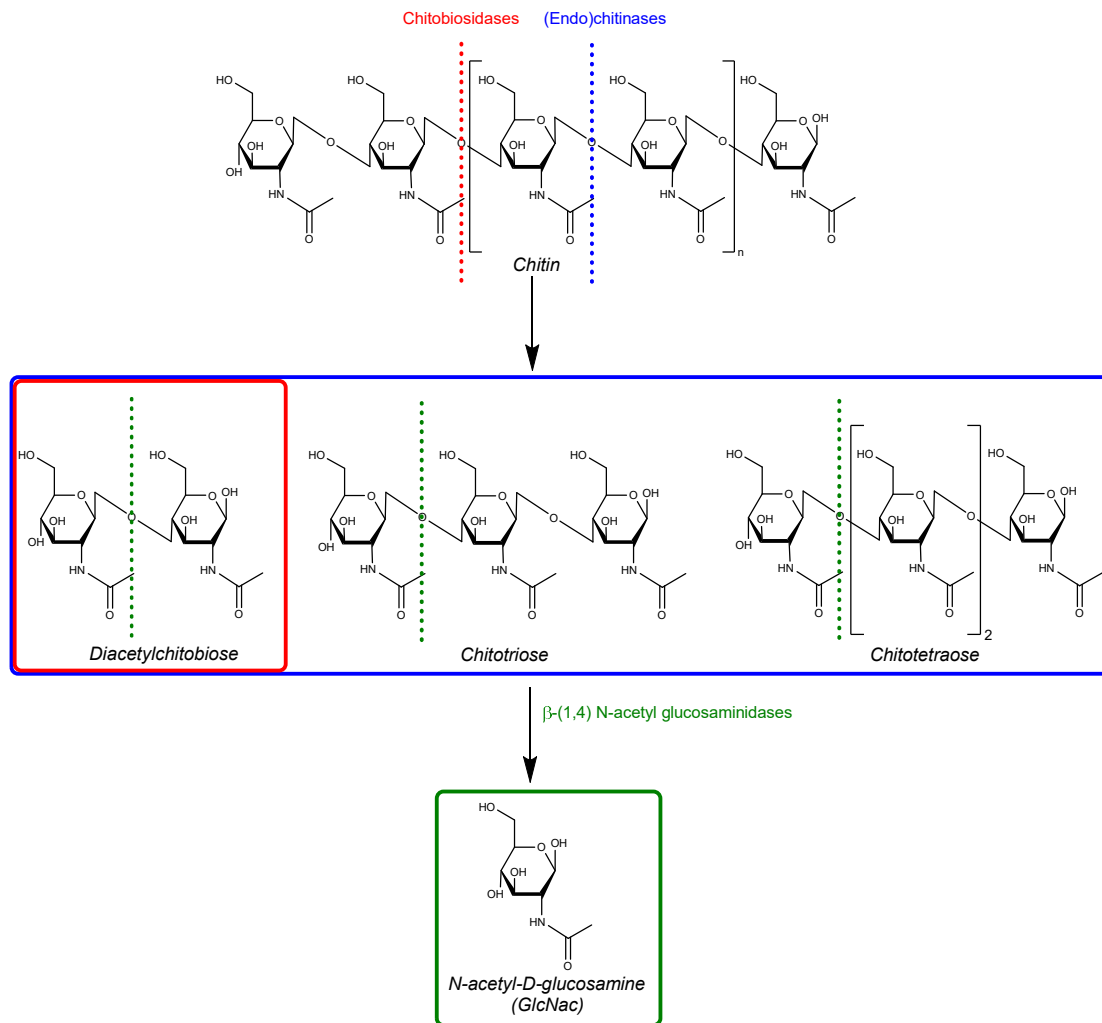


Figure 3: Overview of the chitinolytic system with the three types of chitinases, illustrating the localisation of the glycoside-bond that is hydrolysed by the chitinase (dashed line) and the generated end-products (circled).

Alternatively, based on amino acid sequence similarity, three families of chitinolytic enzymes have been proposed; family 18, 19 and 20 of glycosylhydrolases (GH) (Patil et al., 2000; Rathore and Gupta, 2015; Stoykov et al., 2015). Families 18 and 19 contain chitinases, but they have a completely different amino acid sequence and 3D-structure and thus have most likely been evolved from different ancestors (Rathore and Gupta, 2015; Stoykov et al., 2015). Additionally, they use a different hydrolysis mechanism, in GH18 substrate-assisted catalysis is used, in contrast to GH19, which uses an acid-base mechanism (Stoykov et al., 2015). As a result, there is a difference of substrates between the two families and certain molecules, e.g. allosamidin, can inhibit GH 18 glycosylhydrolases, whilst most family 19 chitinases are resistant (Gooday, 1999). Family 18 is said to be the most diverse in terms of phylogeny containing chitinases from bacteria, fungi, insects, vertebrates and certain plants (Cohen-Kupiec and Chet, 1998; Patil et al., 2000; Stern, 2016). On the other hand, the family 19 glycosylhydrolases have been historically classified as of plant origin, but there have been a few sources reporting GH19 chitinases from bacteria, nematodes and arthropods indicating them to be more widespread than previously thought (Arakane and Muthukrishnan, 2010; Stoykov et al., 2015). GH 18 and 19 have most likely evolved independently from each other (Bussink et al., 2007).

1.3.2. Distribution

A vast array of different organisms, including those that do not contain chitin, produce chitinases for different purposes. In bacteria, chitinases are mainly produced in order to degrade chitin into GlcNac-oligomers to serve as a source of nutrients and energy (Bhattacharya et al., 2007; Rathore and Gupta, 2015). This is especially the case in certain bacteria that often only have chitinous material as sole energy source such as *Vibrio* spp. or marine *Pseudomonas* spp. , which are in large part responsible for the bioconversion of chitin in the water column and sediment of marine ecosystems (Gooday, 1990; Rathore and Gupta, 2015).

Fungal chitinases have multiple biological and physiological roles. Similar to bacteria, they play a role in degrading chitin as a source of energy and nutrients (Cohen-Kupiec and Chet, 1998; Duo-Chuan, 2006). Additionally, they have an essential role in the developmental processes and morphogenesis of fungi such as sporulation, germination and hyphal growth (Duo-Chuan, 2006; Rathore and Gupta, 2015).

Chitinases in plants are mostly considered as pathogenesis-related proteins, they are synthesised in response to an infection by chitin containing organisms and thus play a major role in the defence against fungal and insect pests (Cohen-Kupiec and Chet, 1998; Gooday, 1999; Rathore and Gupta, 2015). It has been reported that they also play an important role during specific processes such as embryogenesis, fruit ripening and ethylene formation (Flach et al., 1992; Rathore and Gupta, 2015). Furthermore, plant chitinases have also been found to play a role in the generation of mycorrhizal associations with certain symbiotic fungi, the digestion of insects by carnivorous plants or to help the plant cope with environmental stressors such as cold and drought (Cohen-Kupiec and Chet, 1998; Rathore and Gupta, 2015; Filyushin et al., 2019).

In ecdysozoans such as insects and nematodes, their primary role is the turn-over of chitinous extracellular matrices such as the cuticle during moulting processes and the formation of the peritrophic membrane around the food bolus (Dahiya et al., 2006; Arakane and Muthukrishnan, 2010). Some protozoans, nematodes and bacteria are able to infect a host by penetrating the peritrophic membrane with their secreted chitinases (Gooday, 1990). Furthermore, chitinases have also been found in the venom and salivary glands as well as the digestive tract of some insects, indicating a possible role in the digestion of the cuticle of their prey (Arakane and Muthukrishnan, 2010; Rathore and Gupta, 2015). For example, in the labial glands of a fungus-growing ant, a complete chitinolytic system has been found, which seems to take part in the degradation of the chitinous material found in the symbiotic fungus (D'Ettore et al., 2002).

Jeuniaux et al. (1982) was the first to suggest a classification of chitinases occurring in the lower vertebrates into three types based on biochemical properties; Type I is found in various organs such as the intestines, pancreas, liver and spleen with a pH optimum of 4 - 4.5 and no activity at pH 1.0 and type II, which is limited to the gastric mucosa and further subdivided. Both type IIa and IIb have a pH optimum at 3.0; however, IIa has a weak activity at pH 1.0, whereas, the opposite is true for type IIb. According to the authors, type I is a 'primitive' type chitinase secreted in a broader range of organs and that evolution towards a more specialised chitinase took place that is more adapted to the acidic environment of the stomach (Jeuniaux et al., 1982). This observation coincides with the hypothesis that in vertebrates a regressive evolution of the biosynthesis of chitinases took place; the invertebrate ancestor has a complete chitinolytic system, i.e. endochitinases and NAGases. In the lower vertebrates, endochitinases are still secreted by the whole gastrointestinal epithelium, but the secretion of NAGases is significantly reduced. By contrast, higher vertebrates possess a more specialised chitinase, but secretion is limited to the gastric mucosa and the ability to synthesise NAGases is lost. Finally, vertebrates with specialised diets, which lack chitinous materials, have entirely lost the property to synthesise chitinases [Figure 4] (Jeuniaux, 1971, 1993; Micha et al., 1973).

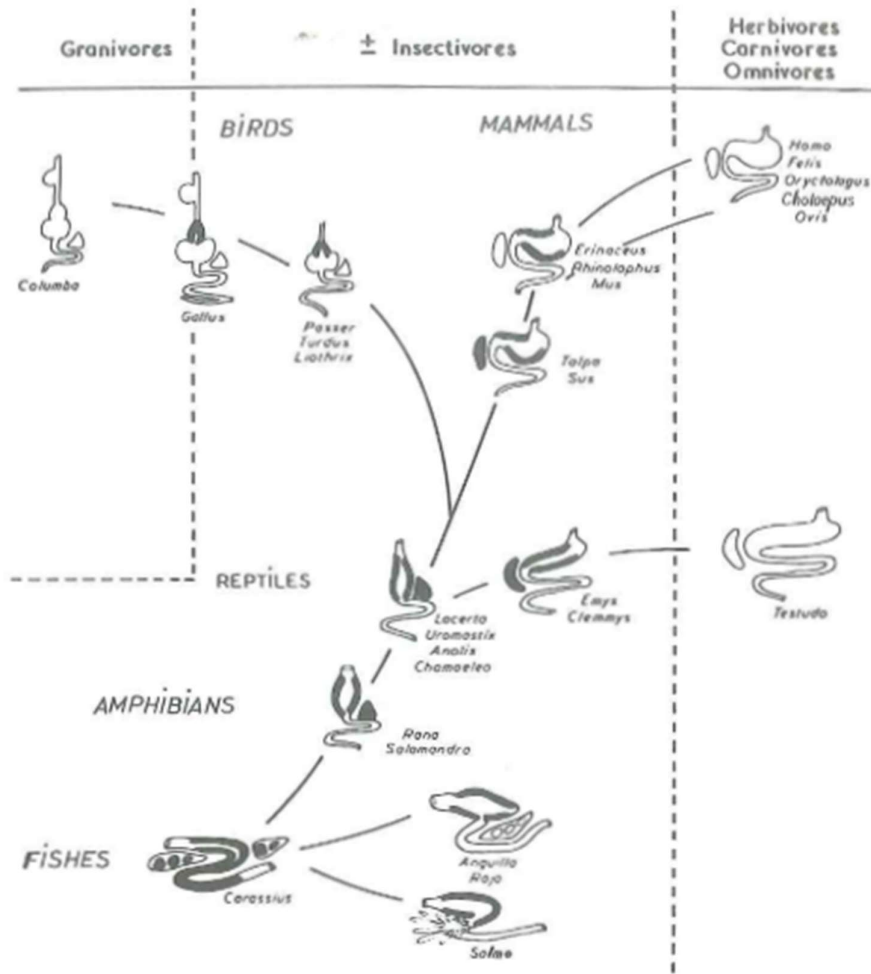


Figure 4: Location of secretion and biosynthesis of chitinase in vertebrates. Black areas mark tissues in which chitinase secretion had been demonstrated by previous studies. From Jeuniaux (1971).

Recent research has indicated the existence of at least three 'types' of chitinase genes, these genes encode for 'true' chitinases, i.e. showing chitinolytic activity towards chitinous substrate with degradation to chito-oligomers, belonging to GH18; chitotriosidase, acidic chitinase and pancreatic chitinase (Oshima et al., 2002a; Stern, 2016).

The first vertebrate chitinase gene to be isolated was chitotriosidase (ChT or chit1) or macrophage-specific chitinase, which was first identified, isolated and cloned from macrophages in humans with Gaucher disease, it has also been cloned in mice and isolated in a crocodilian (*Caiman latirostris*) (Boot et al., 1995; Renkema et al., 1995; Zheng et al., 2005; Siroski et al., 2014). ChT is abundantly synthesised by macrophages when activated by an appropriate stimulus but is also secreted by monocyte-derived cells such as osteoblasts, dendritic cells and Kupffer cells (Stern, 2016). It has been proposed that ChT has a role in innate and acquired immunity against chitinous pathogens such as *Candida albicans*, *Mucor rouxii* and *Cryptococcus neoformans* (van Eijk et al., 2005; Malaguarnera, 2006).

Boot et al. (2001) identified the second vertebrate chitinase gene in rodents and humans which is characterised by an acidic isoelectric point; thus the enzyme was named acidic mammalian chitinase (AMCase, or Chia in non-mammals). The enzyme is relatively abundant in the gastrointestinal tract and due to its acid pH optimum mostly active in the stomach; therefore, it has been suggested that acidic chitinase might play a role in chitin digestion and/or confer resistance against certain parasites such as gastrointestinal nematodes (Boot et al., 2001; Paoletti et al., 2007; Vannella et al., 2016). AMCase is secreted to a lesser extent in the lung of human and mice, where it might be involved in the pathogenesis of asthma, though its exact physiological role remains unclear (Zhu et al., 2004; Boot et al., 2005). Chitinases with similar pH optima and high homology (more than 70%) in their amino acid sequences have been found in cow, chicken and several amphibian species, where it was named toad gastric chitinase (tGCCase) (Suzuki et al., 2001, 2002; Fujimoto et al., 2002; Oshima et al., 2002b). According to Fujimoto et al. (2002), the origin of tGCCase is the oxynticopeptic cells of the stomach. Furthermore, the chitinases isolated from a nine-banded armadillo (*Dasypus novemcinctus*) and a lizard (*Sceloporus undulatus garmani*) might belong to this category, though more research is needed to determine their degree of homology to the other Chia enzymes (Smith and Robbins, 1998; Marsh et al., 2001). Recent studies have shown that in omnivorous species (mouse, chicken, pig and marmoset), Chia is highly expressed in the stomach, has a pH optimum around 2.0, is correlated with the diet and is very resistant against proteases, indicating a dietary role (Ohno et al., 2016; Tabata et al., 2017a, 2017b, 2018, 2019).

Oshima et al. (2002a) identified a third chitinase gene, distinct from Chia and ChT based on amino acid sequence similarity, in the pancreas of *Bufo japonicus*. It was named toad pancreatic chitinase (tPCCase), the enzyme has an optimal pH of approximately 6.0 and is exclusively expressed in the pancreas. It is possible that tPCCase and tGCCase are secreted into the gastrointestinal tract of anurans to facilitate chitin digestion (Fujimoto et al., 2002; Oshima et al., 2002a).

Thus, the possible roles of chitinases in vertebrates can be summarised as follows; biodefense against chitinous pathogens and/or digestion of chitinous materials in their diet.

1.4. ANATOMY OF THE FISH GASTROINTESTINAL SYSTEM

The anatomy of the alimentary canal of fish largely follows the same basic plan, which is shown in *Figure 5* below. In general, the gastrointestinal tract can be divided in a few major compartments, i.e. the mouth and pharynx which form the headgut, the oesophagus and stomach which form the foregut, the intestine or midgut, the rectum or hindgut and related organs such as the liver and pancreas. It is by and large agreed upon that the structure of the regions of the digestive tract of a given species is related to its diet, ontogeny and phylogeny (Fänge and Grove, 1979; Wilson and Castro, 2010).

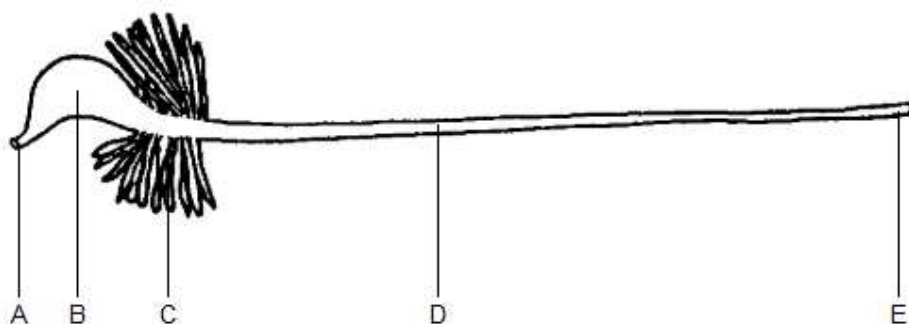


Figure 5: General schematic representation of the GI tract in fish. A: Oesophagus, B: Stomach, C: Pyloric caeca, D: Intestine, E: Rectum. Adapted from Karasov and Hume (2010)

1.4.1. Headgut

The headgut's primary function is acquiring food and the mechanical processing thereof (Wilson and Castro, 2010). Depending on the diet, location of the food items relative to the fish and mode of feeding the following structures can be present and/or modified; the mouth's shape, size and position, gill rakers and marginal and pharyngeal teeth (Aleev, 1969; Wootton, 1990).

1.4.2. Foregut

The foregut is comprised of the intestine and stomach, which serves as temporary food storage and where the chemical degradation of food starts (Bone and Moore, 2008; Wilson and Castro, 2010). In general, the oesophagus of fishes is a short, wide and muscular tube connecting the pharynx to the stomach, or directly to the intestine in agastric fish. The transfer of food is mainly by muscular activity, but in some species the oesophagus is lined with a mucous layer secreted by goblet cells, to facilitate the passage of food whilst also protecting against chemical and mechanical damage (Fänge and Grove, 1979; Horn and Gawlicka, 2001). Generally, there is no clear anatomical demarcation between the oesophagus and stomach in teleost fish. Such a clear demarcation has been found within the Chondrichthyes, where some elasmobranchs possess an esogastric valve (Wilson and Castro, 2010).

Wilson and Castro (2010) classify the stomach into three categories based on their morphology; these are: straight (I-shaped), siphonal (U or J shaped) or cecal (Y-shaped). The straight stomach with an enlarged lumen as it occurs in *Esox* is rather rare and might be in some cases be more indicative of an agastric species. By contrast, the siphonal stomach is the most common shape among the teleosts. Lastly, the cecal stomach allows for the storage of a larger volume of food items. Carnivorous fishes generally have a larger stomach (Wootton, 1990). The stomach is divided into two main regions; the cranial cardiac region, comparable to the fundus and corpus of the mammalian stomach, and the caudal pyloric region, similar to the antrum (Wilson and Castro, 2010; Olsson, 2011). The cardiac region contains the gastric glands, whereas, the pyloric region is mostly devoid of glands except for mucous glands in some fishes (Horn and Gawlicka, 2001; Olsson, 2011). Unlike in mammals, there is only one secretory cell type in the gastric glands: the oxynticopeptic cell. Thus in fish, pepsinogen and hydrochloric acid are secreted by the same cell, whereas in mammals, pepsinogen and acid are secreted respectively by the chief and parietal cell (Bakke et al., 2010; Wilson and Castro, 2010). The pH of gastric juice is approximately between 2.0 and 5.0 and is host to an array of acidic digestive enzymes and hormones (Rust, 2002). Agastric species generally compensate for their lack of stomach by having specialised structures in the headgut or by having a relatively larger midgut to process and digest the food (Wilson and Castro, 2010).

The transition between the pylorus and the midgut may be demarcated by a muscular sphincter and/or a valve-like structure formed by a mucous membrane fold; such an arrangement is also occasionally found within agastric fishes between the oesophagus and intestine (Fänge and Grove, 1979; Wilson and Castro, 2010).

1.4.3. Midgut

In the midgut or intestine, a continuation of the chemical digestion takes place and more importantly, where the primary absorption of nutrients occurs. In some agastric fish, the cranial intestine is dilated and forms an intestinal bulb, which serves as temporary food storage analogous to the stomach; however, it lacks the gastric glands, thus no secretion of pepsinogen or HCl (Wilson and Castro, 2010; Mokhtar, 2017). Agastric species mostly rely on the secretion of pancreatic enzymes such as trypsinogen, amylase and lipase into the intestine for the degradation of macromolecules (Olsson, 2011). The midgut can be arbitrarily divided into three regions; the proximal, middle and distal intestine (Olsson, 2011). The surface area of the intestine is maximised for the absorption of nutrients through the folding of the mucosa and the brush border microvilli. In some teleosts, the surface area may be further increased through convolution, i.e. the looping and coiling of the intestines. However, other teleosts may have a straight and relatively short bowel (Fänge and Grove, 1979).

Research has indicated that carnivorous fish tend to have a relatively shorter gut compared to their omnivorous and herbivorous counterparts (Buddington et al., 1997; German et al., 2010a; German, 2011). It is assumed that herbivorous fish have a longer digestive tract to facilitate a higher intake of low-quality plant material, i.e. high in fibre and low in protein, and to maintain a longer retention time of said material in the gut (Horn and Gawlicka, 2001; German et al., 2010a).

Sixty per cent of all known fish species have blind diverticula connected to the intestine, just posterior of the pylorus, termed pyloric caeca or intestinal caeca (Buddington and Diamond, 1986; Wootton, 1990; Hossain and Dutta, 1996). These blind tubes tend to be better developed in carnivorous/omnivorous fishes and species with a siphonal or cecal shaped stomach; they are absent in agastric species (Hossain and Dutta, 1996; Buddington et al., 1997; Wilson and Castro, 2010). Unlike the caeca found in birds and mammals, which act like fermentation chambers, the function of the pyloric caeca is most likely to increase the surface area of the upper intestine (Buddington and Diamond, 1986, 1987).

1.4.4. Hindgut

The terminal part of the alimentary canal is the hindgut or rectum. The start of the rectum is sometimes demarcated by an ileorectal valve formed by the smooth muscles or a sudden change in diameter and/or folding pattern of the mucosa (Fänge and Grove, 1979; Wilson and Castro, 2010). These characteristics are not always present. Additionally, the rectal tissue is generally quite similar to intestinal tissue; therefore, it can be challenging to discern the midgut from the hindgut in some species (Wilson and Castro, 2010). Hindgut fermentation by symbiotic microbiota may degrade certain food from plant origin in herbivorous species (Bakke et al., 2010; Mountfort et al., 2013).

1.4.5. Liver

The fish liver is a compact, distinct and relatively large organ which may have two or more lobes (Horn and Gawlicka, 2001; Roberts and Ellis, 2012). It is generally ventrally located in the cranial region of the body cavity (Brusle and Gonzalez, 1996). The fishes' liver tissue is comparable to that of other vertebrates, although, on a microscopic level the portal triads and the organisation of hepatocytes in lobules are absent in fish (Rust, 2002; Roberts and Ellis, 2012).

The liver plays a vital role in reproduction (vitellogenesis), blood turn-over, nitrogen catabolism, detoxification, storage (mostly lipids), immunity, and so on (Brusle and Gonzalez, 1996). Its primary digestive function is the production of bile, which is collected and stored in the gall bladder (Brusle and Gonzalez, 1996; Horn and Gawlicka, 2001). The bile duct enters the intestine caudal of the pyloric sphincter or immediately distal of the oesophagus in agastric species (Krogdahl et al., 2011; Olsson, 2011). The bile acids emulsify dietary lipids and fat-soluble vitamins, which allows for the formation of micelles and the action of the main fish lipase (Bakke et al., 2010; Krogdahl et al., 2011).

1.4.6. Pancreas

The fish pancreas is most commonly a diffuse organ, pancreatic nodules are generally scattered in adipose tissue, but can also be found around the mesentery of the pyloric, the subcapsular investment of the spleen, the liver as an external layer around the venae portae hepatis and other sites surrounding the digestive tract (Rust, 2002; Roberts and Ellis, 2012). In other fish species, the pancreas can be present as a discrete organ in close proximity to the intestine (Rust, 2002). Similar to other vertebrates, it consists of two major components; an endocrine component and an exocrine component (Rust, 2002; Caruso and Sheridan, 2011). The endocrine component produces several hormones; glucagon, insulin, somatostatin and peptide YY (Caruso and Sheridan, 2011).

The exocrine pancreas produces digestive enzymes such as proteases, carbohydrases and lipases, which degrade proteins, sugars and fats (Caruso and Sheridan, 2011; Krogdahl et al., 2011). Depending on the fish species, the enzymes are released into the pyloric caeca and/or proximal intestine, or into the bile duct (Bakke et al., 2010). Furthermore, the exocrine pancreas secretes bicarbonate to increase the pH of the chyme to a range of 7.5 to 8.5, this neutralises the acid pH of the digesta coming from the stomach thus protecting the brush border and simultaneously increases the activity of intestinal and pancreatic enzymes (Rust, 2002; Krogdahl et al., 2011).

1.5. DIGESTION OF CARBOHYDRATES

Digestion is a complex and coordinated combination of activities, which can be divided into four categories: physical, chemical, enzymatic and microbial processes. It starts as soon as the food is ingested and lasts until its excretion. Due to the scope of this thesis, the description will be limited to some general concepts and a brief description of the digestion and absorption of carbohydrates by fish, except chitin which will be extensively discussed in a separate section.

1.5.1. Physical processes

The physical processes are mostly limited to the activities in the headgut and muscular contractions of the other parts of the digestive tract. It starts in the mouth where food is often first torn apart, punctured and/or crushed by teeth, buccal pads and/or gill rakers (Krogdahl et al., 2011). For microphagous fish, whose diet consists out of small particles such as plankton, straining and thus concentrating of the ingested material might occur (Rust, 2002). Once food reaches the oesophagus, muscular contractions move the ingesta towards the stomach, if present (Krogdahl et al., 2011). Muscular contractions, in both cardiac and pyloric regions, result in the mixing and grinding of the food and increasing the contact with the gastric enzymes and acid, turning it into chyme (Horn and Gawlicka, 2001). The chyme passes into the intestine, where peristalsis slowly moves and mixes the chyme further down the tract, thus ensuring contact of the nutrients with the brush border enzymes (Rust, 2002).

1.5.2. Chemical processes

Chemical processes are limited to gastric fish. In the stomach, hydrochloric acid is secreted into the lumen by the oxyntopeptic cells, when food is present. Hydrochloric acid is able to break down cellular structures and denature, emulsify and solubilise the main nutrients (Krogdahl et al., 2011). At the same time, HCl converts the inactive pepsinogen into pepsin and causes the pH to be acidic, which increases the activity of gastric enzymes such as pepsin, lipase and chitinase (Rust, 2002).

1.5.3. Enzymatic processes

The principal encountered carbohydrate depends on the diet of the fish. For example, in piscivorous fish, the main carbohydrates are glucose from the prey's blood and glycogen from the muscle and liver tissue. The plant materials eaten by herbivorous fish are likely to contain a certain amount of mono- and disaccharides such as sucrose and fructose, but a more substantial proportion will be polysaccharides such as starch, cellulose and alginates. For insectivorous fish, the main carbohydrates will be chitin from the exoskeleton of insects and crustaceans and trehalose from the haemolymph.

Storage polysaccharides such as amylose, amylopectin and glycogen are hydrolysed by an endogenous enzyme: amylase. In fish, this enzyme is secreted by the pancreas and enterocytes; it breaks down the polysaccharide into di- or oligosaccharides, which are then further cleaved by the intestinal brush border enzymes prior to being absorbed (Krogdahl et al., 2011). According to Kuz'mina and Golovanova (2004), prey carbohydrases can supplement the endogenous enzymes and have a considerable role in the initial digestion of carbohydrates. Disaccharides encountered in food are digested simply by being cleaved by their respective brush border enzymes into their constituent monomers (Horn and Gawlicka, 2001). An overview of the endogenous carbohydrases, their substrate and end-products are supplied in *Figure 6*.

Research indicates that in omnivorous/herbivorous fishes, the activity of amylase is higher when compared to carnivorous fishes (Bakke et al., 2010). Furthermore, a positive correlation was found between the intake of starch and amylase in herbivorous/omnivorous fish; this correlation was absent in carnivorous fish (Karasov and Hume, 2011). Similarly, disaccharidase activity is higher in herbivorous fish than carnivorous fish species (Ugolev and Kuz, 1994).

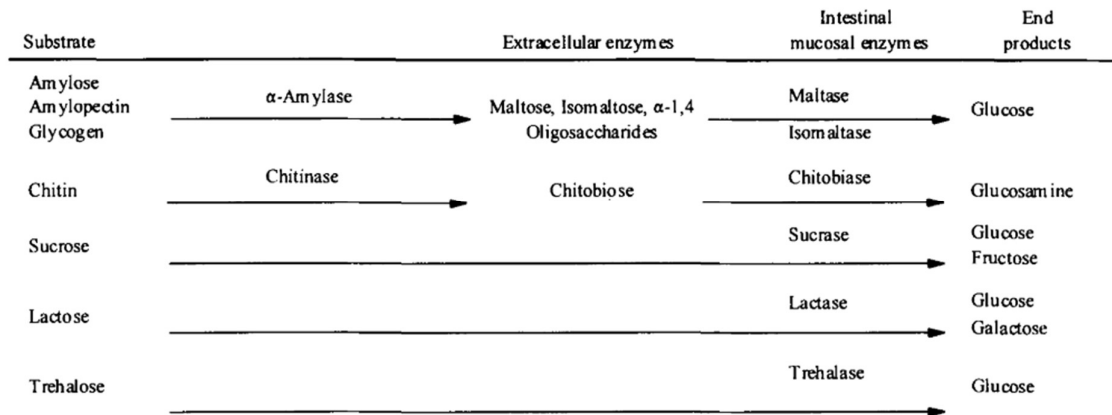


Figure 6: Main endogenous carbohydrases and carbohydrates in fish. Adapted from Karasov and Hume (2011).

1.5.4. Microbial processes

In contrast, one of the main carbohydrates for fish; the structural plant carbohydrate: cellulose, is mostly degraded by exogenous enzymes. Although, there are some fish species such as the grass carp (*Ctenopharyngodon idella*) where a significant amount of the cellulase-activity is endogenous, produced in the hepatopancreas and enterocytes (Das and Tripathi, 1991). It is generally agreed upon that cellulase activity, if present, is mostly from bacterial origin (Stickney and Shumway, 1974; Horn and Gawlicka, 2001). Microbial fermentation mostly takes place in the distal intestine and hindgut; it produces short-chain fatty acids such as acetate, propionate and butyrate, which are absorbed by the fish (Horn and Gawlicka, 2001). The significance of short-chain fatty acids is not yet known, but their role in fishes seems limited based on the observation that in *C. idella*, increasing cellulose inclusion in their diet had little effect on growth (Gao et al., 2010).

1.5.5. Absorption

In contrast to mammals, very little is known about the absorptive processes that take place in the gut of the fish. Dietary carbohydrates are first hydrolysed by their respective carbohydrases into their monomers: D-glucose, D-fructose, D-galactose and GlcNac before they are absorbed by the fish intestine. It appears that fish possess the same mechanisms that are present in mammals, nutrients can enter and exit cells by either simple diffusion or through facilitated diffusion with specialised protein transporters (Bakke et al., 2010). In contrast to mammals, the contribution of paracellular transport of carbohydrates is limited (Krogdahl et al., 2005).

Monosaccharides first have to be transported across the brush border membrane into the enterocyte and subsequently from the cytoplasm across the basolateral membrane into the circulation [Figure 7]. Similar to mammals, glucose and galactose are actively transported against their concentration gradient across the brush border membrane by a Na⁺-dependent transporter molecule, SGLT1, which has been conserved in vertebrate evolution (Horn and Gawlicka, 2001). The SGLT1 driven transport of glucose and galactose has been observed in all currently investigated fishes (Sundell, 2011). Affinity for glucose is the lowest in the pyloric caeca and increases towards the distal parts of the intestine, in contrast to mammals the hindgut of fish is able to absorb nutrients (Krogdahl et al., 2005). Glucose uptake is correlated to the fishes' diet; being the lowest in carnivorous fish, intermediate in omnivorous fish and the highest in herbivorous fish (Krogdahl et al., 2005).

Fructose has been observed to be absorbed by the intestines all examined fish species, but its transport mechanism has not been confirmed yet (Bakke et al., 2010). It is assumed that a GLUT5 transporter molecule allows for the facilitated passage of fructose over the brush border membrane, analogous to mammals (Sundell, 2011). Additionally and also parallel to mammals, GLUT2 transporter molecules on the brush border membrane might allow the facilitated diffusion of monosaccharides, including fructose, into the enterocyte's cytoplasm (Bakke et al., 2010; Sundell, 2011). Whereas, the GLUT2 molecules expressed on the basolateral membrane allow the monosaccharides to exit the enterocyte along their concentration gradient into the bloodstream (Horn and Gawlicka, 2001; Sundell, 2011). Although, several studies have demonstrated the presence of GLUT molecules in the fish intestine, such as GLUT2 and GLUT4, their characteristics, function and exact location remain unknown (Hall et al., 2006; Blanco et al., 2017).

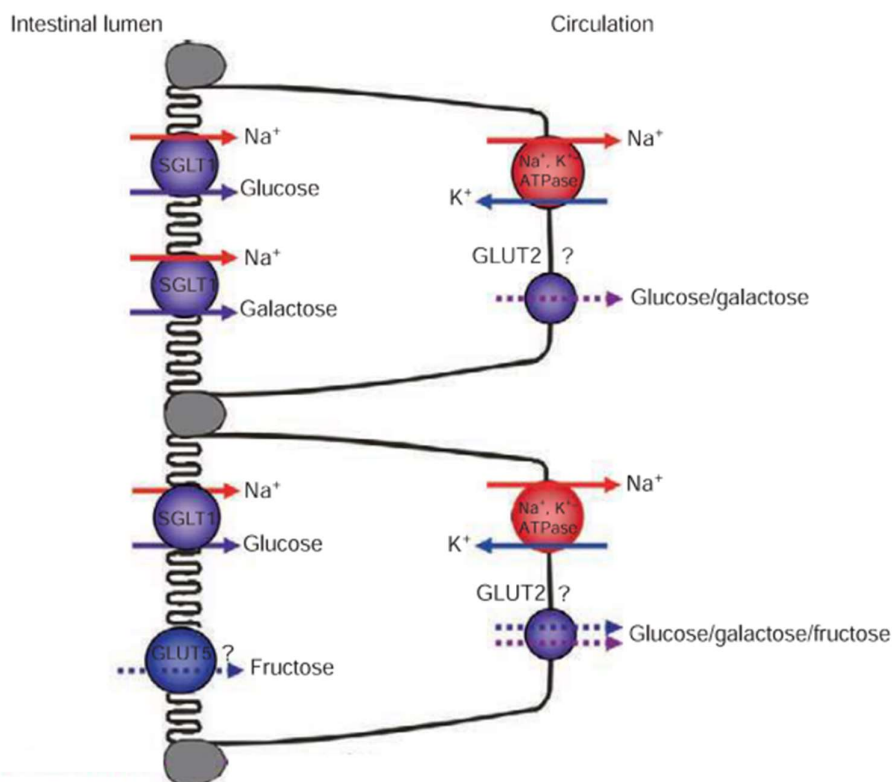


Figure 7: Presumed absorption mechanism for carbohydrates in fish. From Sundell (2011)

1.6. FISH CHITINASES

1.6.1. Origin of chitinase activity in the fish gut

Gooday (1990) proposes three possible origins for the chitinolytic enzyme of the vertebrate's digestive tract: (1) endogenous, i.e. from the animal itself or (2) exogenous, i.e. produced by a commensal gut microbiota or (3) from ingested food.

Jeuniaux (1961) was the first to report the presence of chitinases in the digestive tract of several vertebrates, including several fish species. According to this initial study, chitinases are found not only in the gastrointestinal contents but also in the washed glands of the gastric and intestinal mucosa. Thus the author asserts that it is unlikely that chitinases originate from an exogenous source such as bacteria (Jeuniaux, 1961). Similar findings were made by contemporaries, concluding that based on the localisation of chitinase, it was most likely to be of endogenous origin (Dandrifosse et al., 1965; Micha et al., 1973). It is difficult to make direct comparisons between different studies due to the high variations in methodologies.

However, in general, it can be said that most fishes have high concentrations of chitinases in the digestive tract and that the highest chitinolytic activity is found in the stomach (Yoshida and Sera, 1970; Micha et al., 1973; Fänge et al., 1979; Lindsay and Gooday, 1985; Sabapathy and Teo, 1993; Matsumiya and Mochizuki, 1996; Gutowska et al., 2004).

Other authors have observed that the luminous bacterial population of the typical marine fish gut is dominated by chitinolytic bacteria such as *Enterobacteriaceae* spp., *Photobacterium* spp. and *Vibrio* spp., both the host and the bacteria contributed to the presence of gut chitinases, thus, it was suggested that chitinolytic bacteria in fish gut might have a similar role to the cellulolytic microbiota of the rumen (Okutani, 1966; Hood and Meyers, 1973; O'Brien and Sizemore, 1979; Ruby and Morin, 1979). Chitin might be fermented by the microbiota, predominantly in the hindgut, contributing to vitamin synthesis and other metabolites such as short-chain fatty acids (Kihara and Sakata, 2001a; Rimoldi et al., 2019). Mochizuki and Matsumiya (1981) found high concentrations of lysozyme in several fish species and proposed a possible role of the bacterial enzyme in fish.

Seki and Taga (1963) calculated the theoretical maximal chitinase activity of the chitinolytic bacteria in a pufferfish (*Canthigaster rivulata*) and found that the amount of chitin that could be decomposed in the digestive tract by the bacteria was negligible. They concluded that if a symbiotic relationship is to exist between the fish and the chitinolytic bacteria, the benefit for the host would be the supply of vitamins and other products secreted by the bacteria rather than assisting in chitin digestion (Seki and Taga, 1963). However, because Seki and Taga (1963) estimated the bacterial population size with the plate count method, they might have severely underestimated the number of chitinolytic bacteria. Recent research has indicated that the culturability of intestinal bacteria in marine fish is highly variable and can be extremely low, in the case of *Canthigaster rivulata* a culturability of 0.018 – 2.7 % was found (Sugita et al., 2005).

In an experiment performed by Goodrich and Morita (1977), in which they treated a group of fish (*Enophrys bison* and *Platichthys stellatus*) with chloramphenicol, the authors concluded that fish gut chitinases are exclusively of bacterial origin. However, this view is not supported by later studies performed in other fish species. In similar experiments in which fish were treated with antibiotics, no significant changes in chitinase activity were found despite the decreasing microbiota (Lindsay et al., 1984; E. Danulat, 1986; Kono et al., 1987a; Ramesh and Venugopalan, 1989; Fines and Holt, 2010). It is possible that the earlier results by Goodrich and Morita (1977) were caused by the toxic effects of the antibiotic not only on the bacteria but also on the fish (E. Danulat, 1986).

Others have reported that chitinolytic activity is independent of the number of chitinase-producing bacteria and the amount of dietary chitin present, indicating that chitinase is a constitutive enzyme of endogenous origin rather than an inducible enzyme of bacterial origin (Lindsay and Gooday, 1985; Rehbein et al., 1986). Furthermore, in other studies, high chitinase activities were found in the gastric mucosa of several fish species even though the tissue was carefully washed and isolated or the fish were starved for long periods, thus making it unlikely that the chitinase activity originates from the gastrointestinal microbiota or any ingested food items (Dandriofosse et al., 1965; Colin, 1972; Micha et al., 1973; Lindsay, 1984; Eva Danulat, 1986; Gutowska et al., 2004).

In agastric fishes, such as the holocephalan *Chimaera monstrosa* or the teleost *Chirostoma estor*, the highest chitinase activity was found in respectively the pancreas and the hepatopancreas (Fänge et al., 1976; Pohls et al., 2016). This chitinase is distinct from the one typically located in the stomach, having a more alkaline pH optimum around 8-10, similar to tPCase (Fänge et al., 1976; Oshima et al., 2002a).

Most observations made for the gastric origin of chitinases hold true for the intestine, pancreas and pyloric caeca. The localisation of relatively high chitinolytic activity in the washed glands of the intestinal mucosa and lack of correlation with microbiota, studied after antibiotic treatment, is indicative for an endogenous source (Jeuniaux, 1961; Micha et al., 1973; Danulat, 1986; Fines and Holt, 2010).

In general, there seems to be a tendency towards endochitinase activity being the highest in the stomach and progressively decreasing towards the hindgut (Colin, 1972; Micha et al., 1973; Fänge et al., 1979; Rehbein et al., 1986; Matsumiya and Mochizuki, 1996; Gutowska et al., 2004; Kakizaki et al., 2015). Additionally, endochitinase activity is the highest in the anterior part of the stomach, i.e. the cardia, and relatively lower in the posterior, i.e. pylorus (Yoshida and Sera, 1970; Lindsay et al., 1984).

Although the cellular origin of endochitinases has not been determined yet, the patterns of activity indicate that the oxynticopeptic cells are the most likely candidate, similar to tGCCase in anurans (Fujimoto et al., 2002). In contrast, a reversed pattern can be discerned for NAGase activity. Its activity is often the highest in either the intestine or accessory digestion organs such as the liver or hepatopancreas (Matsumiya and Mochizuki, 1996; Kakizaki et al., 2015; Pohls et al., 2016). NAGase activity in the intestine tends to follow the general pattern of fish brush border enzymes, i.e. progressively increasing from the proximal intestine, peaking in the middle gut and then slightly decreasing (German et al., 2010b, 2015). However, in some of the investigated species the highest recorded chitin activity was found in the hindgut (German and Bittong, 2009; German et al., 2015; Jhaveri et al., 2015). For example, in the bonnethead shark (*Sphyrno tiburo*) the spike of NAGase activity in the colon, including its contents, is strongly indicative for a microbial origin (Jhaveri et al., 2015).

Kurokawa et al. (2004) were the first to isolate fish chitinase genes in the olive flounder (*Paralichthys olivaceus*), where they identified three different genes; flounder chitinase 1 (fChi1), flounder chitinase 2 (fChi2) and flounder chitinase 3 (fChi3). The fChi1 and fChi2 are considered acidic fish chitinases as they are mostly expressed in the gastric glands of the stomach; furthermore, their amino acid sequence bears similarity with the AMCases. The third chitinase is expressed by various tissues and might be considered as a flounder chitotriosidase (Kurokawa et al., 2004). Since then authors have identified fish chitinases in the stomach of several Osteichthyes and have proposed, based on phylogeny, three unique chitinase groups; acidic fish chitinase-1 (AFCCase-1), acidic fish chitinase-2 (AFCCase-2) and fish chitinase-3 (FCCase-2) (Ikeda et al., 2013; Kakizaki et al., 2015; Ikehata et al., 2016; Pohls et al., 2016; Watanabe et al., 2018). AFCCase-1 and AFCCase-2 have differing degradation patterns, AFCCase-1 preferentially hydrolyses the second glycosidic bond from the non-reducing end of chitin, whereas the latter prefers the third glycosidic bond (Ikehata et al., 2016). These results indicate that at least some fish have an efficient enzymatic chitin-degrading system, which utilises three different endogenous chitinases.

In conclusion, there is little doubt left that the majority of chitinolytic activity in the gastrointestinal tract of fish is of endogenous origin. However, a number of chitinolytic bacteria have been found in several fish species, with the bacterial population often the densest in the hindgut region (Hamid et al., 1979; MacDonald et al., 1986; Sakata and Koreeda, 1986; Banerjee et al., 2016). Additionally, certain fish have high populations of chitinolytic bacteria in their gut, e.g. 98.8 % of intestinal *Vibrio* spp. isolates in the marine *Paralichthys olivaceus* and over 90 % of intestinal *Aeromonas* spp. in the freshwater *Cyprinus carpio* (Sugita et al., 1999; Sugita and Ito, 2006). Thus, a significant contribution by bacterial fermentation cannot be excluded yet. Based on the currently available research, it seems likely that the bacterial fermentation of chitin is dependent on the fishes' diet. For example, in lepidophagous catfish, it is presumed that bacterial endosymbionts play an important role in the digestion of chitin (Gosavi et al., 2018, 2019). In the carnivorous *O. mykiss*, it was indicated that hindgut bacteria could make use of chitin as an energy substrate, resulting into either an increased synthesis of short-chain fatty acids (SCFA) or reduced utilisation of the existing SCFA (Kihara and Sakata, 2001a). In contrast, there was little to no production of SCFA by the intestinal microbiota in the detritivorous *O. niloticus* and omnivorous *C. carpio* in the presence of chitin (Kihara and Sakata, 1997, 2001b).

1.6.2. Role of fish chitinases

Based on the observation that in many fishes, most having a diet that includes chitinous arthropods or molluscs, chitinases are widely distributed, a digestion-related role for chitinases has been proposed for them (Karasov and Hume, 2011). Their exact role in digestion remains unclear; however, two major roles can be discerned in the current literature, namely a nutritive or disruptive role. Their primary function is likely to vary between species. The chitinases that are primarily found in lymphomyeloid organs presumably have a role in the immune system. Additionally, the chitinases predominantly found in the alimentary tract might also protect against chitinous pathogens such as nematodes, bacteria or fungi. A schematic overview of the proposed functions of chitinases can be found in the flowchart below [Figure 8].

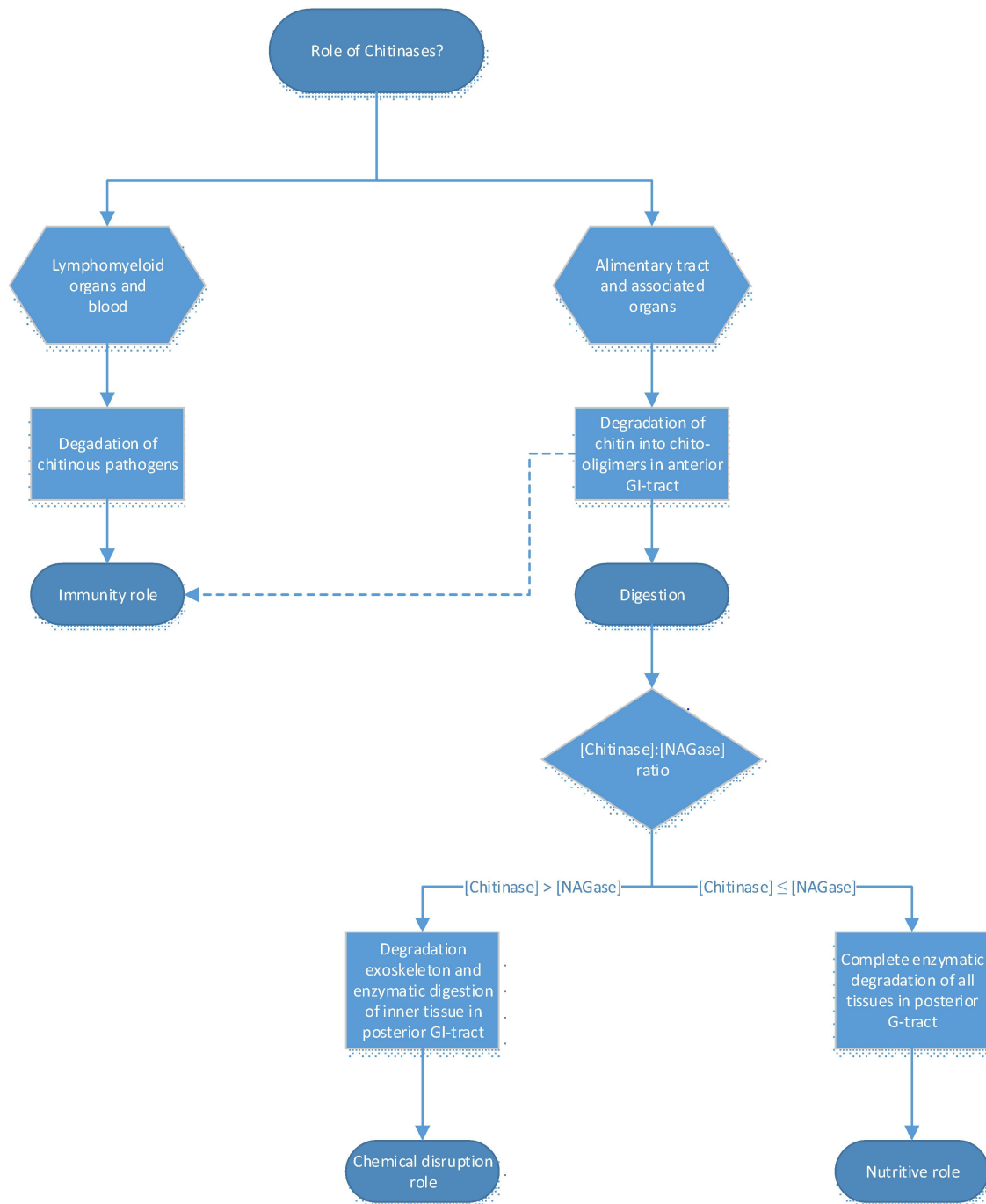


Figure 8: Flowchart with proposed roles of fish chitinases based on current knowledge

1.6.3. Nutritive role of the chitinolytic system

The chitinases have a nutritive role, i.e. they facilitate the enzymatic degradation of chitin into its monomer GlcNac, which is subsequently absorbed and serves as an energy source (Fänge and Grove, 1979; Jeuniaux, 1993; Karasov and Hume, 2011). An important prerequisite for this complete degradation is the presence of a complete chitinolytic system, i.e. the presence of endochitinase and NAGase in sufficient quantities is necessary in order to achieve a total break-down of chitin (Jeuniaux, 1993; Karasov and Hume, 2011).

To reiterate, most fishes possess chitinases which are primarily found in the stomach, where they degrade chitin into chito-oligomers. By contrast, NAGases are mostly found further down the digestive tract, in the mid- and/or hindgut (Fänge and Grove, 1979; Clark et al., 1988; Jeuniaux, 1993; Gutowska et al., 2004; Abro et al., 2014). In a few fishes, chitinases and NAGases were found in equivalent concentrations, thus it can be assumed that in these species, a complete degradation of chitin is achieved (Eva Danulat, 1986; Matsumiya and Mochizuki, 1996; Gutowska et al., 2004; Pohls et al., 2016; Ikeda et al., 2017b).

It has been observed that in some fish species, e.g. *Gadus morhua*, chitin is highly digestible (~ 90%) in *Gadus morhua*, where only a small fraction of the ingested chitin was recovered from intestinal contents (9%) and faeces (2,4%) (Danulat, 1987). Furthermore, several studies have been conducted to investigate the effects of chitin inclusion in the fish's diet. Effects on growth could be indicative of chitin utilisation and metabolism. Two types of study can be identified; on the one hand, those that directly include purified chitin in the diet [Table 1] and on the other hand, those where fish meal was partially replaced by an alternative protein source, which contains a significant amount of chitin, e.g. insects, crustaceans or sea-food by-products. Studies have indicated a positive result on growth when chitin is supplemented in the fish feed (Kono et al., 1987b; Om et al., 2003; Moren et al., 2006; Mohan et al., 2009). Similarly, the addition of recombinant or bacterial chitinase in the diet leads to increased growth performance in fish (Zhang et al., 2012, 2014). According to Nakagawa (2007), chitin supplementation leads to an accelerated protein deposition whereby protein deposition in the muscle is increased, and that of lipid decreased in the liver; thus, improving growth.

Chitin has a caloric value of ~ 17.1 kJ.g⁻¹ (comparable to protein), and the chitinous exoskeleton of some arthropods can contain over 20% of the available energy (Karasov, 1989; Gutowska et al., 2004). Thus it is likely that chitin, which some consider the dominant carbohydrate of the natural fish diet, might have a significant contribution towards energy intake and as a nitrogen source (Danulat, 1987; Krogdahl et al., 2005). The absorption of GlcNac has been documented in several fish species; furthermore, in the case of *Scylliorhinus canicula*, it was found that GlcNac was absorbed more readily than glucose (Alliot, 1967; Pérès et al., 1973). However, at the time of writing, little is known about the uptake and further metabolic pathway of GlcNac. It is likely that in fish, GlcNac is absorbed unaltered through facilitated diffusion, analogous as observed in rats (Tesoriere et al., 1972). Possibly, GlcNac is absorbed through the GLUT2 transporter, which was shown to have a high affinity for glucosamine in *Xenopus* (Uldry et al., 2002). For the further metabolism, Krogdahl et al. (2005) propose two metabolic pathways [Figure 9]; on the one hand, GlcNac could be passed into a synthetic pathway requiring GlcNac or glucosamine. On the other hand, the glucose-core of GlcNac can be funnelled into glycolytic intermediates through a pathway of phosphorylation into GlcNac 6-phosphate, deacetylation into glucosamine 6-phosphate and finally deamination and isomerisation resulting into fructose 6-phosphate and ammonia (Krogdahl et al., 2005).

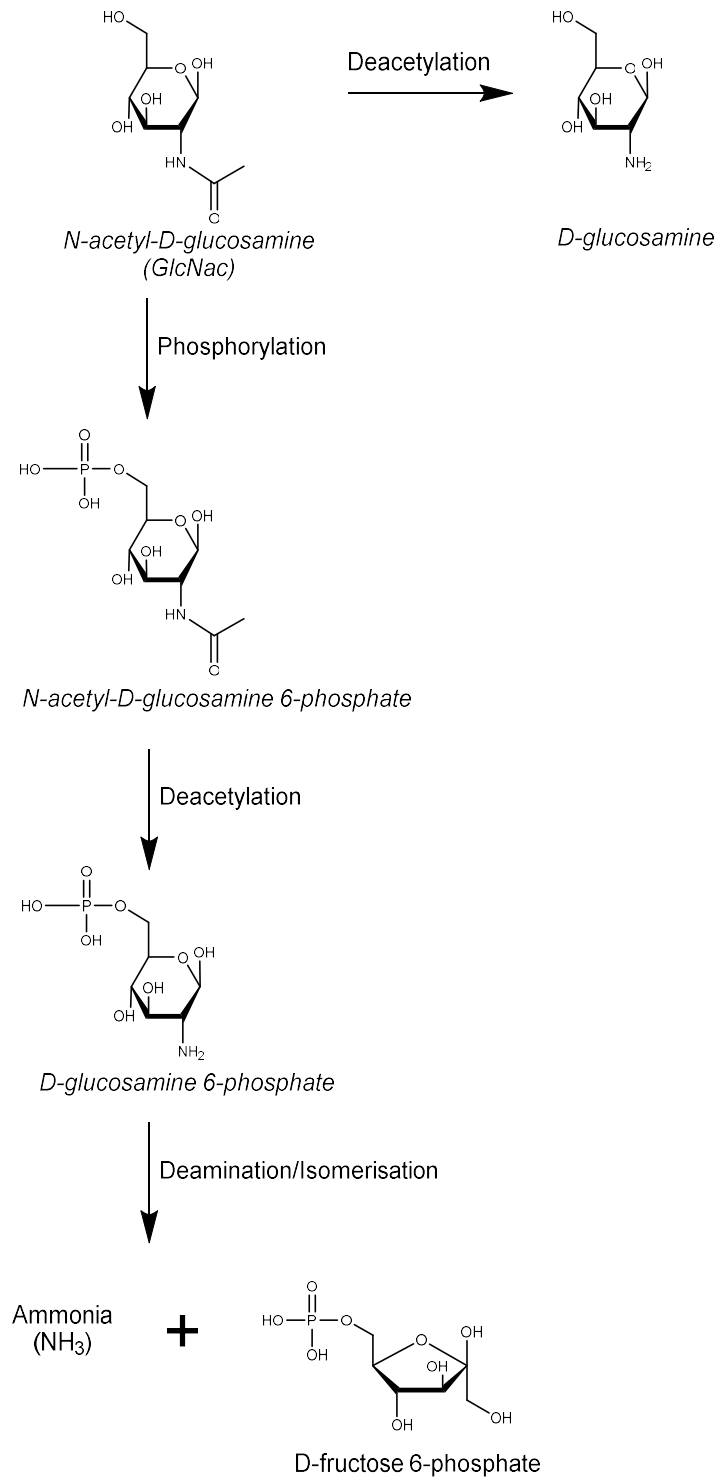


Figure 9: Proposed metabolic pathways for GlcNac in fish.

Other studies with chitin inclusion in the diet of fish, e.g. rainbow trout (*O. mykiss*), hybrid tilapia (*Oreochromis niloticus* X *Oreochromis aureus*) and so on, have produced contradictory results, where fish growth was negatively impacted (Lindsay et al., 1984; Shiao and Yu, 1999; Gopalakannan and Arul, 2006; Olsen et al., 2006; Kroeckel et al., 2012). It is possible that in these species, the chitinolytic activity is lower or the chitinolytic system is incomplete; thus, they are unable to digest chitin.

The research found that digestibility of chitin by rainbow trout and three cichlid species was practically zero (Buddington, 1980; Lindsay et al., 1984). Other studies have reported that chitinous dinoflagellates remained wholly undigested in several *Oreochromis* spp. (Spataru, 1978; Spataru and Zorn, 1978). Furthermore, in the case of *O. niloticus*, negligible NAGase and moderate chitinase activity were found in the digestive tract (Molinari et al., 2007). Thus, in the case of cichlids, it can be assumed that chitin is not digested due to a lacking chitinolytic system. Remarkably, *O. mykiss* has one of the highest chitinase activities recorded amongst fish and a substantial NAGase activity in the gastrointestinal tract, yet chitin digestibility is poor (Okutani et al., 1967b; Micha et al., 1973; Lindsay et al., 1984). It is possible that the potent chemical treatment and denaturation of the chitin used in *O. mykiss* trials negatively impacted its digestibility; additionally, the α -chitin which was prepared from the dorsal carapace of *Cancer pagarus*, is more resilient than the other polymorphs commonly found in insects and crustaceans (Lindsay et al., 1984). However, in a trial with *Pagrus pagrus* in which fish meal was partially replaced with marine crab meal (*Chaceon affinis*), growth significantly increased, yet no difference was found with the river crab meal (*Procambarus clarkii*) (García et al., 2010). This could indicate that chitin digestibility is dependent on its origin.

Alternatively, the negative effect of chitin on growth may also be explained by differences in molecular weight and degree of acetylation (Abro et al., 2014). Inclusion of dietary chitin might also result in impaired overall nutrient digestibility and/or disturbed nutrient absorption (Shiau and Yu, 1999; Tharanathan and Kittur, 2003; Abro et al., 2014). Remarkably, in juvenile rainbow trout was increased with 6% chitin inclusion in the diet, despite the digestive tract only reaching enzymatic 'maturity' after the first three to five weeks of feeding (Kawai and Ikeda, 1973; Lindsay, 1985; Lellis and Barrows, 2000). Thus, chitin inclusion might increase growth under certain circumstances, changing with ontogeny, or indirectly enhance performance through mechanisms different from digestion such as immunostimulation.

A lot of studies have examined the effects of partial or total fish meal replacement with insects, krill, or other chitin-rich material (Henry et al., 2015). The results of these have been inconclusive, even when results of the same species of fish, fed the same chitin-rich source, are compared, contradictions appear. For example, in studies by Sealey et al. (2011), St-Hilaire et al. (2007) and Dumas et al. (2018), growth of *O. mykiss* decreased when the fish meal was partially replaced with *Hermetia illucens* prepupae larvae. However, other trials have replaced up to 50% of fish meal with *H. illucens* prepupae larvae without any significant effects on *O. mykiss* growth (Stadtlander et al., 2017; Cardinaletti et al., 2019; Józefiak et al., 2019). A commonality shared by studies with results which find that growth is depressed after fish meal replacement is that chitin, and the fish's lacking capability for digestion thereof is often blamed as the cause, although the problem could have originated from other components. For example, Yoshitomi et al. (2006, 2007) reported that at higher replacement levels of fish meal with krill (*Euphausia superba*), fluoride accumulation is responsible for the decreased growth. Additionally, some authors may have incorrectly concluded that fish are unable to digest chitin (Rust, 2002). Kroeckel et al. (2012) found reduced growth in turbot after fish meal replacement with *H. illucens* and reported that this was due to the lack of detectable chitinase. However, they measured enzymatic levels in the mid-intestine, instead of the stomach.

Table 1: Overview of available literature on dietary chitin inclusion trials in fish

| Investigated fish species | %chitin in diet | Chitin source | Quantity | Study duration | Effect compared to control | Reference |
|---|-----------------|--|---|----------------|--|------------------------------|
| <i>Oncorhynchus mykiss</i> | 4, 10 or 25 | <i>Cancer pagurus</i> (dorsal carapace) | 2% Biomass/day | 12 weeks | Growth was significantly depressed at all inclusion levels | Lindsay et al., 1984 |
| <i>Pagrus major</i> | 10 | NA | 20-25% Bodyweight/day | 30 days | Improved growth | Kono et al., 1986 |
| <i>Anguilla japonica</i> | 10 | NA | 5-10% Bodyweight /day | 30 days | Improved growth | Kono et al., 1986 |
| <i>Seriola quinqueradiata</i> | 10 | NA | 20% Bodyweight /day | 30 days | Improved growth | Kono et al., 1986 |
| <i>Oreochromis niloticus x Oreochromis aureus</i> | 2, 5 or 10 | NA | 5% Bodyweight /day | 8 weeks | Higher %chitin-inclusions coincided with growth depression | Shiau and Yu., 1999 |
| <i>Oncorhynchus mykiss</i> | 6 | <i>Euphausia superbia</i> (Ground exoskeleton) | Initially 6% Bodyweight /day End-of-trial 4,5% Bodyweight /day | 15 weeks | Improved growth | Lellis and Barrows., 2000 |
| <i>Acanthopagrus schlegeli</i> | 10 | NA | hand-fed 2 times/day to apparent satiation | 50 days | Improved growth | Om et al., 2003 |
| <i>Cyprinus carpio</i> | 1 | Shrimp shell waste | ad libitum | 90 days | Depressed growth compared to control | Gopalakannan and Arul., 2006 |
| <i>Schizothorax richardsonii</i> | 2 | NA | 5% Bodyweight /day | 8 weeks | Improved growth | Mohan et al., 2009 |
| <i>Tor putitora</i> | 2 | NA | 5% Bodyweight /day | 8 weeks | No significant difference | Mohan et al., 2009 |
| <i>Salmo salar</i> | 1, 2 or 5 | <i>Pandalus borealis</i> (exoskeleton) | 2,5%biomass/day | 13 weeks | Decreased growth | Karlsen et al., 2015 |
| <i>Gadus morhua</i> | 1, 2 or 5 | <i>Pandalus borealis</i> (exoskeleton) | Fed in slight excess | 13 weeks | No significant effects | Karlsen et al., 2015 |
| <i>Hippoglossus hippoglossus</i> | 5 | <i>Pandalus borealis</i> (exoskeleton) | Fed in slight excess | 13 weeks | No significant effects | Karlsen et al., 2015 |

1.6.4. Chemical disruption

Alternatively, because of the typically higher chitinolytic activity in the stomach of fishes and the acidic optima for chitinases, combined with the observation that in some species the chitinolytic system is limited by the NAGase activity, it has been proposed that the purpose of chitinases is the disruption of prey and mechanical breakdown in the stomach prior to the passage into the intestine (Lindsay, 1984; Seiderer et al., 1987; Gutowska et al., 2004; Karasov and Hume, 2011). In this scenario, chitinases would facilitate the breakdown of the chitinous cuticle of prey, in order to allow the inner tissue to be reached by the fish's digestive enzymes. Additionally, high chitinolytic activity in the stomach and intestine might degrade particles that otherwise could cause blockage, thus maintaining intestinal flow (Lindsay, 1984). This offers an explanation as to why certain fish species have relatively high chitinase activity in the absence of NAGase.

Multiple studies have established a correlation exists between the level of chitinase activity and the extent to which a certain fish species is able to physically disrupt prey (Lindsay, 1984; Gutowska et al., 2004). I.e. fish that have certain structures in the headgut (e.g. gill rakers or mandibular teeth) or longer intestines, which respectively allow the fish to mechanically break up the food or permit longer retention times, have relatively lower chitinase activities. Whereas, fishes that lack such modifications and thus tend to swallow prey whole have relatively high chitinase activity (Lindsay, 1984; Gutowska et al., 2004; German et al., 2010a). Conversely, no correlation was found between diet and chitinase/NAGase activity, even though, such a relationship might be expected in the case where chitinolytic enzymes play a nutritive role (Nagayama and Saito, 1968; Colin, 1972; Micha et al., 1973; Lindsay, 1984; Benmouna et al., 1986; Rehbein et al., 1986). In the 13 carnivorous marine fish species, studied by Gutowska et al. (2004), it was found that chitinase activity was more related to the intestinal length than diet. The fish species with shorter intestines had a higher chitinolytic activity (Gutowska et al., 2004).

However, others have found that fish, with diets containing higher amounts of chitin, have higher chitinolytic activities (Goodrich and Morita, 1977b). Another study found that *G. morhua* consuming crustaceans have higher chitinolytic activities than those that are fed a fish diet (Eva Danulat, 1986). According to German et al. (2010a), such a correlation may be observed when two closely related species, with different dietary specialisations, are examined.

1.6.5. Immunity

Research has found that high chitinase and lysozyme activity occurs in the blood plasma and lymphomyeloid tissues (except for the thymus) of several Chondrichthyes and teleosts (Fänge et al., 1976, 1980; Lundblad et al., 1979; Molinari et al., 2007). Some have suggested that the high enzymatic activity in lymphomyeloid tissue and blood might indicate a role in defence against chitinous pathogens, such as parasitic nematodes, arthropods and fungi (Alexander, 1985; Alexander and Ingram, 1992). It was demonstrated that in the turbot, *Scophthalmus maximus*, lysozyme, chitinase and NAGase activities were exceptionally high within the leucocytes, enabling these cells to digest potential fungal pathogens (Manson et al., 1992). Additionally, Leiro et al. (1997) demonstrated that chitinase in turbot serum modifies the surface glycoproteins of fungal spores, reducing uptake by splenic macrophages, which might further spread the infection in case they fail to kill the ingested spores. Simultaneously, superoxide production in macrophages was significantly increased, thus ingested fungal spores, are destroyed more effectively (Leiro et al., 1997).

In male *Lampetra japonica*, the secretion of chitinases in the liver increased after fungal/bacterial challenge (Liu et al., 2009). The expression-pattern of chitinase3 in *Danio rerio* embryos implies a dual function in innate immunity and digestion (Teng et al., 2014).

Zhang et al. (2012) found that in *Epinephelus coiodes*, bacterial LPS induced an up-regulation of chitinase1 and chitinase2 as well as the protein secretion of chitinase1 in the spleen, both enzymes can bind to chitin and inhibit bacterial growth. GlcNac is a major constituent of LPS; thus, the overexpression of chitinases after bacterial challenge may be considered as a specific immune response. Similarly, in *S. maximus* there was an up-regulation of the chitinase genes in mucosal surfaces after bacterial challenge, indicating their role in the prevention of pathogen attachment and entry in mucosal immunity (Gao et al., 2017).

Curiously, high NAGase activities, sometimes exceeding that of the digestive tract, have been found in the lymphomyeloid tissues and blood of several fish species (Fänge et al., 1979, 1980; Ueno et al., 1988; Yuan et al., 1991). It was suggested that NAGase (and chitinase) might facilitate or inhibit certain immunological responses by modifying surface antigens (Fänge et al., 1980).

Research has also indicated that chitin has an immunostimulatory role through the non-specific modulation of the cellular and humoral immune system (Nishimura et al., 1984; Kawakami et al., 1998). There is some evidence that chitin increases protection against bacterial infections, *Vibrio anguillarum* and *Photobacterium damselae* subsp. *piscicida*, when injected intravenously (Sakai et al., 1992; Kawakami et al., 1998). In vitro studies have demonstrated that fish leukocytes are stimulated by chitin and its derivatives (Hoffman et al., 1997; Cuesta et al., 2003). Similar results were obtained in vivo; the innate immune responses, i.e. respiratory burst, phagocytic and cytotoxic activities were boosted after intraperitoneal injection (Esteban et al., 2000). Similar results were observed after oral administration of chitin (Esteban et al., 2001). Furthermore, dietary chitin can increase survivability against bacterial and parasitic infections, though it seems a minimal level of chitin inclusion is required, or no effect is observed (Choudhury et al., 2005; Gopalakannan and Arul, 2006; Kumar et al., 2019).

Additionally, dietary inclusion of chitin in *O. mykiss*, *G. morhua* and *Salmo salar* modulate the intestinal microbiota resulting in higher bacterial diversity, which in turn might reduce the risk of enteric pathogen invasion by outclassing them in nutrient acquisition or colonisation of the gut tract (Askarian et al., 2012; Zhou et al., 2013; Huyben et al., 2019).

1.6.6. Link to morphometry

According to German et al. (2015), fishes show similarities in patterns across diversification of gut morphology and digestive enzyme activities. Carnivorous fish generally have a shorter alimentary tract and higher aminopeptidase activity when compared to herbivorous fish. Herbivorous fish, on the other hand, tend to have longer guts and elevated amylase activity. Omnivorous fish usually take up the middle space of the spectrum, though exceptions are known to occur (German et al., 2015). Based on these phylogenetically informed studies, a similar pattern might arise, when comparing closely related species with differing levels of chitin-intake. Such a study was performed by (German et al., 2010a), in closely related minnow species. It was found that the more insectivorous minnows had a shorter intestinal tract and higher chitinase activity than their herbivorous counterparts (German et al., 2010a).

2. THE AIM OF THE STUDY

Based on the currently available literature; it may be assumed, that most, if not all, fish possess endogenous chitinases and are thus able to hydrolyse chitin to varying extents. However, the role of chitinases and the extent to which chitin can be utilised as a source of nutrition and energy is still largely unknown. It is likely to be different between species, depending on their dietary preference and phylogeny.

The goal of this study was to determine the chitin utilisation of several wild freshwater fish species found in the same area. If chitin utilisation is present, the extent to which will be compared between the species and an attempt will be made to investigate if any relationship exists between the capacity of chitin utilisation and morphometry or diet. A total of five native fish species will be investigated. Three of which are phylogenetically related, a fourth which is not related but endemic to the region and a fifth, which is an introduced species.

2.1. OBJECTIVE 1: GUT MORPHOLOGY AND MORPHOMETRY

Dissection and measuring will give us an insight into the general gross morphology of the gut. Macroscopically identifiable food items can indicate the diet of the fish and to what extent certain items are digested. Data can be used to test if it matches the general patterns that have been described in fish. Such as the relationship between diet and intestinal length. Additionally, it will allow us to investigate if a relationship between chitinase activity and morphology exists.

2.2. OBJECTIVE 2: STABLE ISOTOPE ANALYSIS.

This will provide us with a better insight into which prey and how efficiently it was digested by the fish. By measuring stable isotopes in predator and comparing them with the already known SIA – profile of the prey items. The data from SIA can be used to establish if a relationship between chitinase activity and diet exists.

2.3. OBJECTIVE 3: METABOLOMICS

By analysing to what extent chitin-related end-products are found, i.e. N-Acetyl-D-glucosamine and N-Acetyl-D-Glucosamine-6 in different compartments of the fish, we can establish to what extent chitin digestion (i.e. chitinase-activity) and metabolisation take place.

2.4. OBJECTIVE 4: GENE EXPRESSION.

If chitinase producing genes could be isolated and their transcripts in the alimentary tract measured. We would be able to estimate endogenous chitinase activity in the fish and how these are distributed in the intestine. Combined with results from metabolomics, these results could have indications on the efficiency of these endogenous enzymes or on the presence of a chitinolytic bacterial biota.

3. MATERIAL AND METHODS

3.1. STUDY SITE AND FISH COLLECTION

From November to December 2018 fish were caught in Lake Titicaca, in close proximity to Isla del Luna, Bolivia. All fish were caught by local fishermen using a nylon benthic gillnet with mesh size 5 – 25 mm. In total, 225 fish were collected for this study. Upon capture, fish were either killed with blunt force to the head or collected in a bucket with water from the lake for transportation to Institución Pública Desconcentrada de Pesca y Acuicultura (IDP-PACU), San Pablo de Tiquina, Bolivia for sterile sample collection. Fish were then identified until species level, following species were determined; the endemic *Orestias albus* (n=66), *Orestias ispi* (n=61), *Orestias luteus* (n=57), and *Trichomycterus rivulatus* (n=20) and the introduced *Oncorhynchus mykiss* (n=22).

3.2. MORPHOMETRIC DATA OF THE INVESTIGATED FISH

Fish were then measured with a calliper, with a closed mouth, (standard length [SL] \pm 1 mm) and weighed (body mass [BM] \pm 0.01 g). Fish were dissected as follows. First, a ventral incision through the skin, muscle and the peritoneum from the anal fin to the operculum. Second, the operculum and pectoral fin, including the pectoral girdle were removed, thus exposing the gills. Third, an incision from the top of the gills, parallel to the dorsum, towards the tail and then down to the anal fin. Finally, the body wall was removed, exposing the body cavity [See Figure 10].

The intestinal tract was then removed by cutting the oesophagus as cranially as possible and cutting the rectum as caudally as possible, i.e. where it was attached to the body wall. The morphology of the alimentary tract was macroscopically observed in situ. The guts were then carefully uncoiled, to prevent stretching, and measured with a calliper (gut length [GL] \pm 1 mm) and macroscopically investigated. Moreover, in gastric species, the intestinal length was determined by measuring from the pylorus to the anus (intestinal length [IL] \pm 1 mm). Finally, the guts, including their contents, were weighed (gut mass [GM] \pm 0.01 g). Gut contents were not systematically investigated, though some short notes were made on the macroscopically identifiable food items in order to establish an idea of the fish's diet. In *O. mykiss* this was done by making an incision along the alimentary tract. In the other species, the gut was cut into different parts; gut contents could then be stripped from them, using a pair of anatomical tweezers.

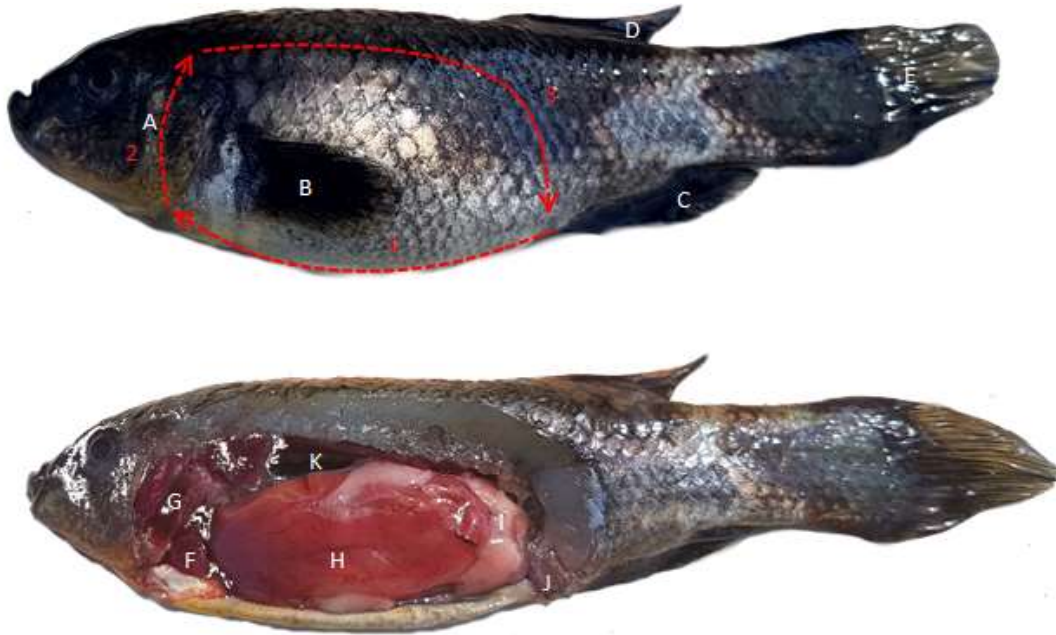


Figure 10: *Orestias albus*, Upper photo showcasing the three dissection steps in red. Lower: After removal of part of the left body wall A = Operculum, B = Pectoral fin, C = Anal fin, D = Dorsal fin, E = Caudal fin, F = Heart, G = Gills, H = Liver, I = Adipose tissue, J = Hindgut; K = Swim bladder

3.3. STATISTICAL ANALYSIS OF MORPHOMETRIC DATA

To compare the gut size among the investigated species, two somatic digestive indexes were used, Zihler's index [ZI] and relative gut length [RGL]. Both have been used successfully in comparisons of the gut size in the available literature. Moreover, the relative gut mass [RGM], which is the ratio of GM to BM was also compared to examine the quantity of food between the different fish species.

Formula Zihler's index:

$$ZI = \frac{GL [mm]}{10 \times \sqrt[3]{BM(g)}}$$

Formula relative gut length:

$$RGL = \frac{GL [mm]}{SL [mm]}$$

Formula relative gut mass:

$$RGM = \frac{GM [g]}{BM [g]}$$

Statistical analysis of the somatic indexes was performed by one-way ANOVA, using the RStudio Desktop software package (version 1.2.1335). In order to accept the results of the Anova, two conditions need to be met; the residuals need to be normally distributed, and the variance within each of the species needs to be equal. Normality of the residuals was tested with the Shapiro-Wilk normality test. Homogeneity of the variance was tested with the Bartlett test. If the residuals were not normally distributed, but the homogeneity of the variance was present, a Box-Cox lambda transformation was performed in order to normalise the data and thus residuals. If the residuals were not standardised after Box-Cox transformation and/or homogeneity of the variance could not be established, the non-parametric Kruskal-Wallis test was used to analyse the data instead. The data are respectively presented as the mean \pm SEM and mean and range. P values < 0.05 were considered to be statistically significant.

3.4. STABLE ISOTOPE ANALYSIS [SIA]

For SIA, muscle tissue and bone tissue from several fishes were collected. For the muscle tissue, > 1 cm² of white muscle was collected from the left lateral side of the fish, just caudal of the above-described incision. Simultaneously, bone tissue was collected from the same fish by removing several vertebrae from the spine. Tissue samples were then individually collected in Eppendorf tubes and left to dry out in the sun with an open lid. Samples were shipped to Belgium and further dried in an oven at $\pm 60^\circ$ C at the Department of Nutrition, Genetics and Ethology, Ghent University, Belgium. Tissue samples were then processed according to the following protocol:

- Demineralisation (only applied to bone samples)
 1. Insert bone samples together with 0.5 N hydrochloric acid into glass bottles. The volume of HCL should be at least ten times higher than that of the bone sample.
 2. Seal the glass bottles and let it rest for 24 hours in the refrigerator at 4° C.
 3. Remove HCL solution and put into an appropriate disposal container
 4. Rinse the samples three times with distilled water.
 5. Dry the samples for at least 24h in an oven at 60° C
- Lipid extraction (both muscle and bone tissue)
 1. Insert the tissue sample together with a 2:1 chloroform:methanol solution into glass bottles.
 2. Seal the glass bottles and let it rest for 24 hours at room temperature.
 3. Remove chloroform:methanol solution and put into an appropriate disposal container.
 4. Repeat steps 1-3 twice.
 5. Rinse the tissue samples with distilled water three times.
 6. Dry the samples for at least 48 hours in an oven at 60° C

Samples were then collected into a 96 well-plate and shipped by dr. Muñoz Saravia to an appropriate lab for analysis

3.5. METABOLOMICS

On two separate occasions during the study period, several fish (n=5, per species) were transported to Institución Pública Desconcentrada de Pesca y Acuicultura, San Pablo de Tiquina, Bolivia. IDP-PACU provided their laboratory facilities for this part of the research. Fish were transported live in buckets, filled with water from Lake Titicaca, to the laboratory. The water was intermittently replaced to ensure enough oxygen for the fish was present. In IDP-PACU, fish were placed in a container with ample oxygen. Fish were individually weighed ([BM] ± 0.01 g) and measured with closed mouths ([SL] ± 1 mm) after euthanasia by blunt force to the head. Fish were then aseptically opened according to the same dissection technique used for the collection of the morphometric data [Figure 10]. Blood was directly aspirated from the ventricle and collected on the dried blood spot card according to instructions of the manufacturer. Afterwards, the gut was aseptically removed and uncoiled. The alimentary tract was measured ([GL] ± 1 mm) and weighed ([GM] ± 0.01 g). Afterwards, the gut was cut into separate, depending on the fish these regions corresponded with different compartments of the alimentary tract as outlined in *Table 2*.

Due to the limited size of *Orestias ispi*, thus limited quantity of gut contents, it was decided not to divide the alimentary tract. Likewise, in *Trichomycterus rivulatus*, no compartmentalisation was made due to the limited amount of gut contents that were present in the collected individuals.

Table 2: Summary of sampling sites of investigated fish species

| | Region 1 | Region 2 | Region 3 |
|--|-----------------------------------|---|----------|
| <i>Oncorhynchus mykiss</i> | Oesophagus and stomach | Proximal intestine and associated pyloric caeca | Hindgut |
| <i>Orestias agassi</i> | Proximal half of alimentary tract | Distal half of alimentary tract | NA |
| <i>Orestias luteus</i> | Proximal half of alimentary tract | Distal half of alimentary tract | NA |
| <i>Orestias ispi</i> | Alimentary tract | NA | NA |
| <i>Trichomycterus rivulatus</i> | Alimentary tract | NA | NA |

Gut contents from these different regions were then aseptically collected in sterile single-use falcon tubes. Gastro-intestinal tissue was collected for gene expression analysis, see infra. Gut contents were centrifuged at 5 000 RPM for 30 minutes. The supernatant was aspirated with single-use syringes and collected onto the dried blood spot cards according to the manufacturer's instructions. Dried blood spot cards were sent to UMC Utrecht, the Netherlands for further analysis. They performed the Direct Infusion – Mass Spectrometry (DI – MS) and performed the data analysis with the R-based MetaboShiny platform developed by Joanna Wolthuis (Wolthuis et al., *submitted*).

3.6. GENE EXPRESSION

After collecting the gut contents, the alimentary tract was carefully rinsed with distilled water to remove any left-over gut contents. Tissue samples were collected from three different parts of the gastrointestinal tract, with the exception of *O. ispi*, due to its limited size, only two tissue samples were collected. Additionally, in all species, a part of liver tissue was collected.

Table 3: Locations of tissue collection for gene expression analysis

| | Region 1 | Region 2 | Region 3 |
|--|------------------------------------|---|----------------------------------|
| <i>Oncorhynchus mykiss</i> | Stomach | Proximal intestine and associated pyloric caeca | Hindgut |
| <i>Orestias agassi</i> | Proximal third of alimentary tract | Middle third of alimentary tract | Distal third of alimentary tract |
| <i>Orestias luteus</i> | Proximal half of alimentary tract | Middle third of alimentary tract | Distal third of alimentary tract |
| <i>Orestias ispi</i> | Proximal half of alimentary tract | Distal half of alimentary tract | NA |
| <i>Trichomycterus rivulatus</i> | Stomach | Proximal intestine | Hindgut |

Tissue samples were collected in sterile Eppendorf tubes, with RNA*later* solution. They were shipped to the Department of Nutrition, Genetics and Ethology, Ghent University, Belgium and stored at -40°C. However, due to the time-frame needed to get the results, it was decided not to analyse these samples.

4. RESULTS

4.1. MACROSCOPIC ANATOMY OF THE INVESTIGATED LAKE TITICACA FISH

4.1.1. *Oncorhynchus mykiss* (rainbow trout)

Oncorhynchus mykiss has a short, muscular oesophagus, which dilates caudally to form the stomach. The oesophagus is connected to the swim bladder through a pneumatic duct. The stomach is siphonal or J-shaped. It is comprised of a longer dorsal zone; the corpus or cardiac part of the stomach, a curved middle transitional area and a shorter ventral part; the pyloric part of the stomach. The intestine is a simple loop, consisting of an ascending and a descending part. The ascending intestine is characterised by the presence of multiple caeca. At the most proximal part, pyloric caeca were the longest and could be found around the full circumference; distally they were mostly limited to the lateral and ventral sides of the intestine. The caeca were oriented towards the caudal side. The descending intestine slightly increased in width to form the rectum.

The liver is a dark-red compact structure, limited to the cranial part of the visceral cavity. The gallbladder could be found on the visceral surface of the liver; its contents were emptied into the proximal part of the intestine, i.e. at the level of the pyloric caeca, through the common bile duct.

The alimentary tract of the trout was generally full of freshwater amphipods, such as *Hyalella*, which was the only macroscopically identifiable structure. The largest fraction of amphipods was found in the stomach, though a considerable amount was found throughout the gut, except for the oesophagus and pyloric caeca. *Hyalella* in the stomach were the least digested, often intact amphipods could be found. In the distal parts of the intestine, considerable amounts of exoskeletons of the *Hyalella* could be recognised.

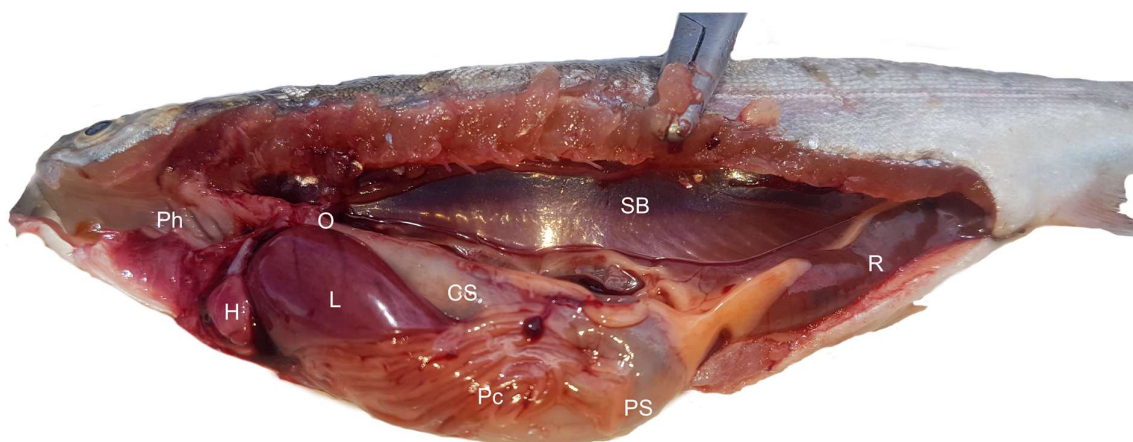


Figure 11: Left-lateral view alimentary canal in-situ. Ph=Pharynx, H=Heart, O = Oesophagus, L = Liver, CS = Cardiac part of the stomach, PS = Pyloric part of the stomach, PC = Pyloric caeca, SB = Swim bladder, R = Rectum

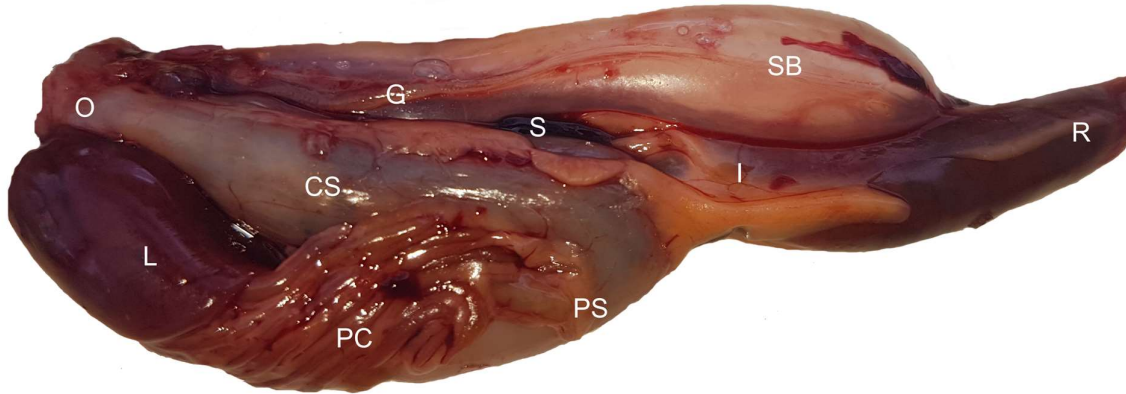


Figure 12: Left-lateral view of alimentary tract 'removed *en masse*' O=Oesophagus, CS = Cardiac part of stomach, PS = Pyloric part of stomach, PC = Pyloric caeca, L = Liver, G = Gonads (developing), S = Spleen, SB = Swim bladder, I = intestine, R = Rectum

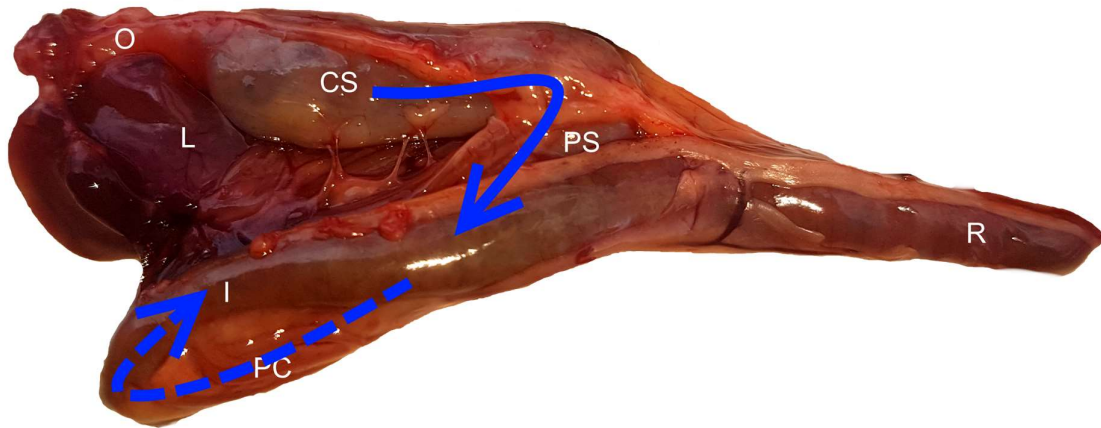


Figure 13: Medial view of alimentary tract 'removed *en masse*' Blue arrows show direction of ingesta, O = Oesophagus, L = Liver, CS = Cardiac part of stomach, PS = Pyloric part of stomach, I = Intestine, R = Rectum

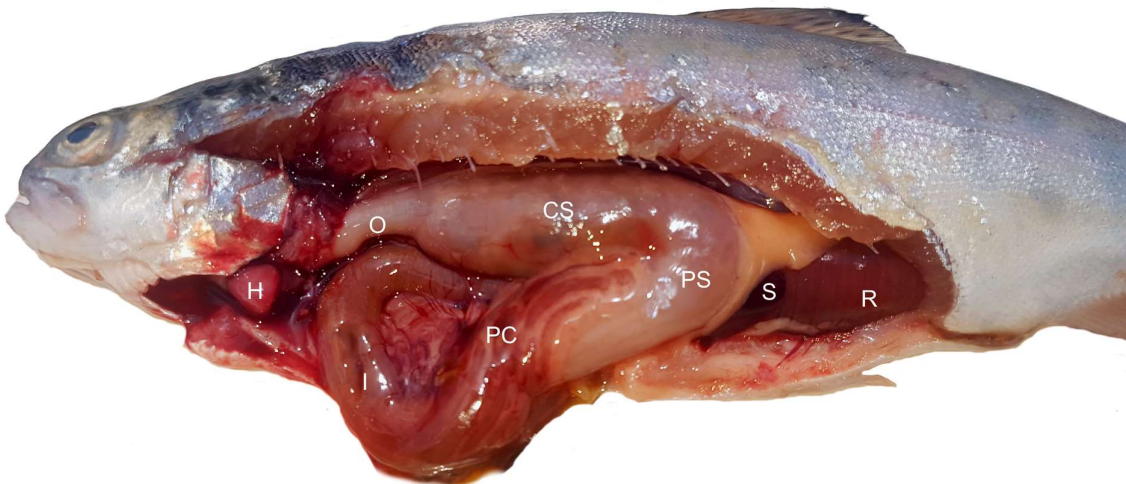


Figure 14: Left-lateral view after removal of the liver. H = Heart, O = Oesophagus, CS = Cardiac part of stomach, PS = Pyloric part of stomach, I = intestine, S = Spleen, R = Rectum

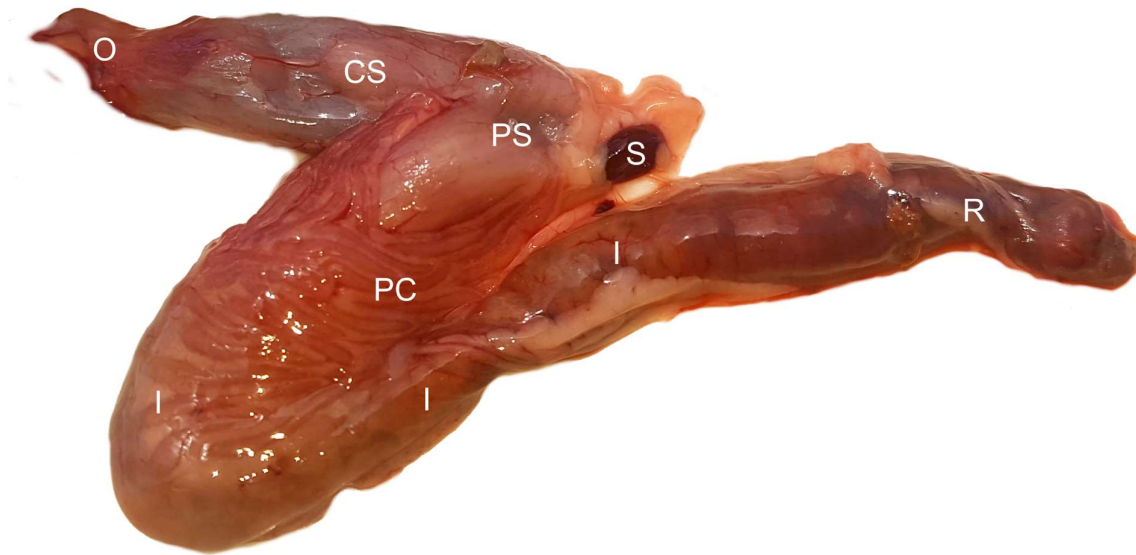


Figure 15: Left-lateral view of the partially uncoiled alimentary tract. O = Oesophagus, CS = Cardiac part of stomach, PS = Pyloric part of stomach, PC = Pyloric caeca, I = Intestine, R = Rectum

4.1.2. *Orestias* spp. (pupfish of Lake Titicaca)

Three species were examined during the study; *Orestias luteus*, *Orestias agassi* and *Orestias ispi*. However, because of the high similarities between the gross morphology of the alimentary tract, their anatomy will be described on a genus-level.

The general form of the gut of *Orestias* spp., especially when uncoiled, is a simple straight tube without any visible differences between the different compartments and the absence of the pyloric caeca. The short oesophagus is connected with the swim bladder through a clearly present pneumatic duct. The stomach seems to be absent in this genus. cursory tests with pH-strips found no acidic zones in the gut; additionally, the gallbladder empties into the proximal part of the gut through a common bile duct, and pyloric caeca are absent. These observations are indicative that the observed *Orestias* spp. are agastric. The oesophagus opens into the intestine, demarcated by the entrance of the common bile duct. The intestine is characterised by the presence of four loops, which were named: the hepatic loop, proximal loop, middle loop and distal loop. In some agastric fish, the proximal intestine is enlarged to form the intestinal bulb. However, no such structure was found within the examined *Orestias* spp. The last straight part of the intestine transitions into the rectum, located ventrally of the gonads, without clear demarcation.

All examined *Orestias* spp. have a red liver, which extends from the cranial part of the visceral cavity to the caudal part, covering the gonads completely. It appears to be bilobular; a smaller ventral lobe covers the lateral and dorsal aspect of the oesophagus and first segment of the intestine. The relatively bigger dorsal lobe is bordered by the first segment of the intestine on the dorsal side, the hepatic loop on the caudal side, the second segment of the intestine on the ventral side and by the caudal border of the operculum on the cranial side. Although the liver is relatively long, it only takes up a limited space of the visceral cavity because it is rather flat. On the parietal surface, the gallbladder can be found in a groove near to the proximal loop, which is emptied into the intestine, distally from the pneumatic duct. The spleen is also located on the parietal surface, near the distal loop.

Unsurprisingly, gut contents were considerably different among the *Orestias* spp. In *O. luteus*, the identifiable food items were the empty mollusc shells of freshwater microgastropods, *Heleobia*. The shells were wholly undigested by the fish. Unlike the other two *Orestias* spp., the relatively wider head permits the *O. luteus* to eat these snails. Additionally, several exoskeletons of aquatic insects and amphipods could be identified, in a similar state as those found in *O. mykiss*. In *O. agassi*, only exoskeletons of amphipods could be identified. Similar to *O. luteus*, these exoskeletons were more-or-less in the same state as those found in the rainbow trout.

A recurring pattern in older individuals of both species was that generally the proximal half of the gut was more empty compared to the distal half. One possible explanation for this is that the less or non-digestible parts, i.e. the *Heleobia* shell or *Hyaella* exoskeleton could cause intestinal blockage, make transit of chyme more difficult in the distal part. The effect was more pronounced in *O. luteus*, despite the relatively larger diameter of the gut; due to the size of the *Heleobia* shell, which is probably more prone to causing a blockage. Lastly, in *O. ispi* no food items, except for fish eggs in a few individuals, were able to be macroscopically identified. Presumably, this fish's diet consists largely of zooplankton.

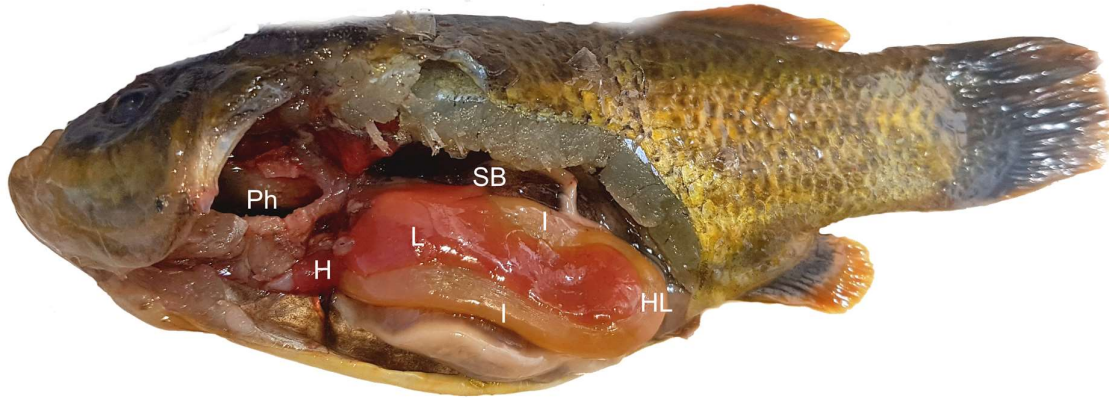


Figure 16: Left-ventrolateral view alimentary tract in-situ (*Orestias luteus*). Ph = Pharynx, H = Heart, L = Liver, SB = Swim bladder, I = Intestine = Hepatic loop.

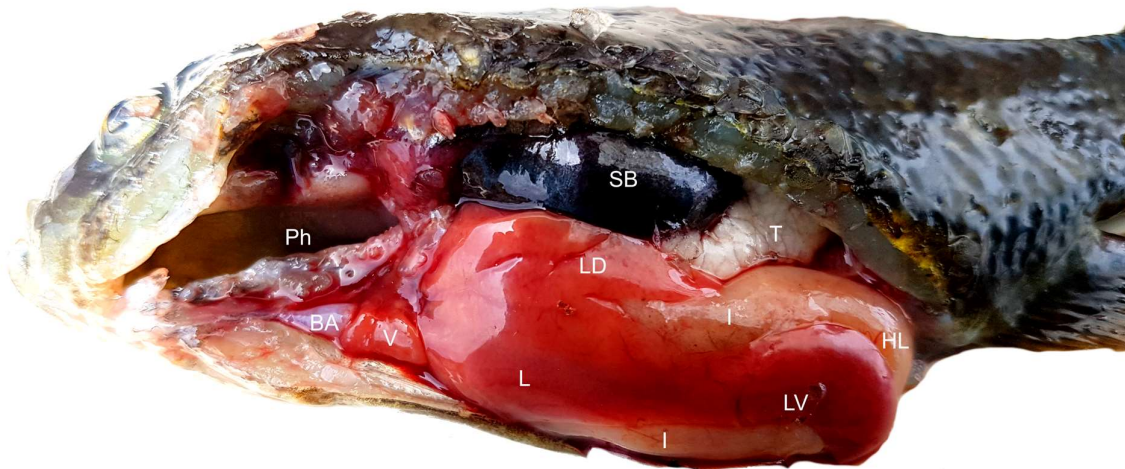


Figure 17: Left-ventrolateral view of alimentary tract in-situ (*Orestias luteus*). Ph = Pharynx, BA = Bulbus arteriosus, V = Ventricle, L = Liver with dorsal lobe (LD) and ventral lobe (LV), SB = Swimbladder, T = Testes, I = Intestine, HL = Hepatic loop

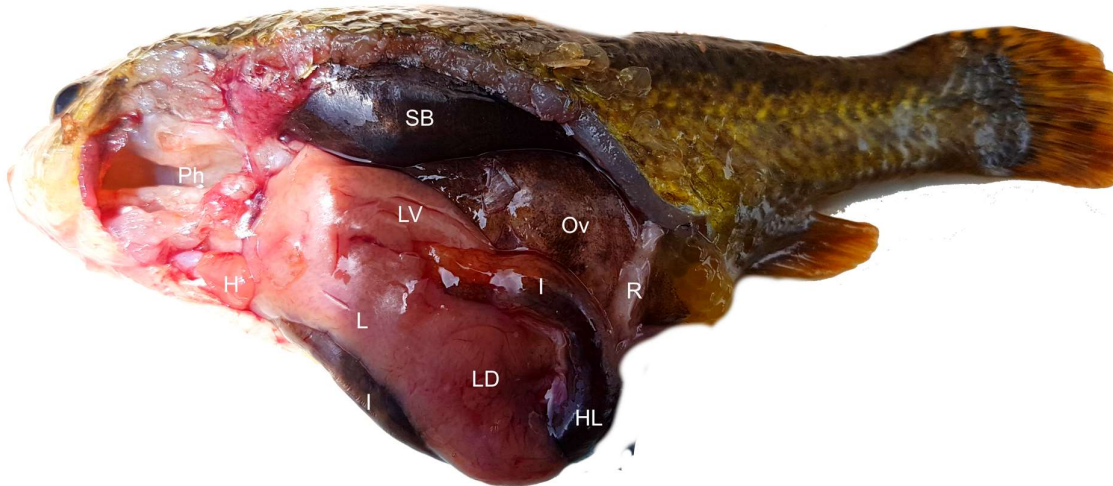


Figure 18: Left-ventrolateral view of the alimentary tract in situ (*Orestias luteus*). Ph = Pharynx, H = Heart, L = Liver with dorsal lobe (LD) and ventral lobe (LV), SB = Swimbladder, Ov = Ovaries, I = Intestine, HL = Hepatic loop, R = Rectum

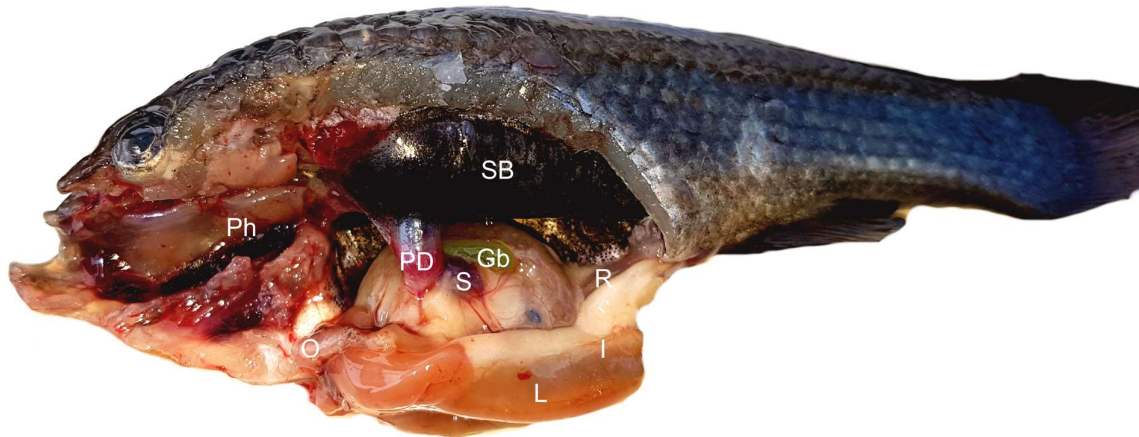


Figure 19: Left lateral view (*Orestias agassii*), the gonads were removed. Ph = Pharynx, O = Oesophagus, PD = Pneumatic duct, Gb = Gallbladder, S = Spleen, I = Intestine, L = Liver, R = Rectum

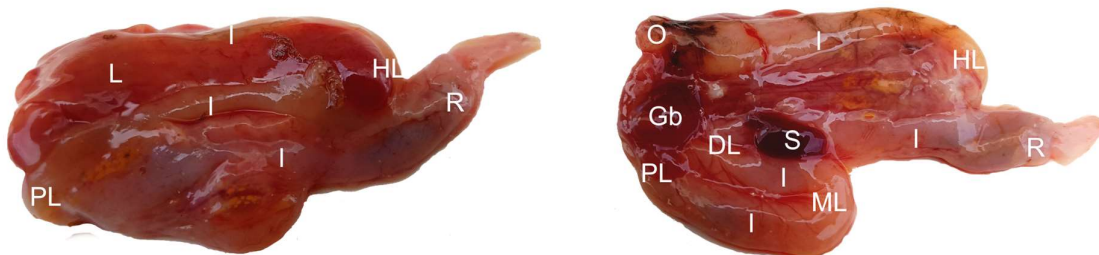


Figure 20: Alimentary tract "removed en masse" (*Orestias luteus*). Left: "parietal surface". Right: "visceral surface". O = Oesophagus, I = Intestine, L = Liver, HL = Hepatic loop, PL = Proximal loop, ML = Middle loop, DL = Distal loop, R = Rectum, Gb = Gallbladder, S = Spleen

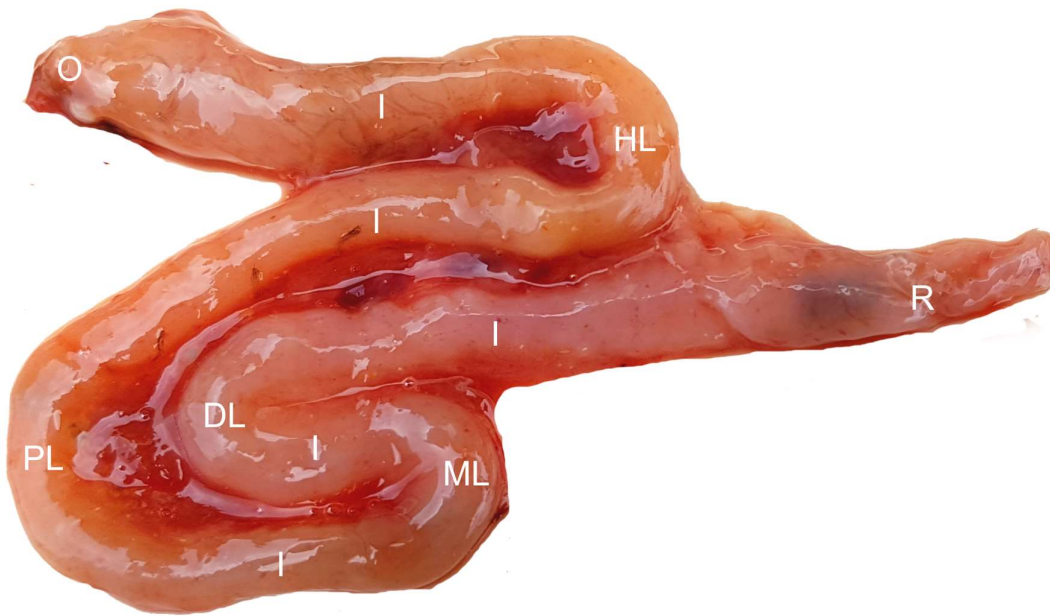


Figure 21: Left-lateral view of the alimentary tract (*Orestias luteus*). O = Oesophagus, I = Intestine, HL = Hepatic loop, PL = Proximal loop, ML = Middle loop, DL = Distal loop, R = Rectum



Figure 22: Left-lateral view of the uncoiled alimentary tract (*Orestias luteus*). O = Oesophagus, I = Intestine, R = Rectum

4.1.3. *Trichomycterus rivulatus* ('el suche')

Similar to the *Orestias spp.* the alimentary tract, in its uncoiled state, appears as a simple straight tube as it lacks pyloric caeca or any other distinguishing characteristics. The alimentary tract starts with a short and wide oesophagus, which dilates into a J-shaped stomach, comprised of a longer dorsal cardiac part and shorter ventral part. The oesophagus is connected through a pneumatic duct to the relatively small and round swim bladder, which is limited to the cranial part of the visceral cavity. On the whole, the gut is characterised by a relatively small diameter; although there appear to be local dilatations in the alimentary tract, these are caused by the presence of ingested amphipods, which locally stretch the gut. There are no pyloric caeca present to mark the transition between the intestine and stomach, but in some individuals, there was a well-developed constriction separating these regions. The common bile duct enters into the midgut distally from this constriction if present. The intestine itself loops three times; proximal loop, middle loop and distal loop. The topography of the gut is maintained through the firm mesentery and ligaments, which connect the different compartments. Ultimately, it transitions into the rectum, which lies ventrally of the gonads, without any external demarcations.

The liver is a red, compact structure, which is located in the first part of the visceral cavity. It is firmly attached to the alimentary tract through ligaments. The gallbladder is relatively small and is located on the visceral surface.

Characteristically to all investigated *T. rivulatus*, was their generally empty intestinal tract. Only in a few species a small amount, ranging between one and four, of *Hyalella* exoskeletons were recovered. These were mostly intact, difficult to remove without cutting through the intestine and causing local distensions of the gut, thus possibly leading to an intestinal blockage. Other contents were of plant-origin such as algae and plant detritus.

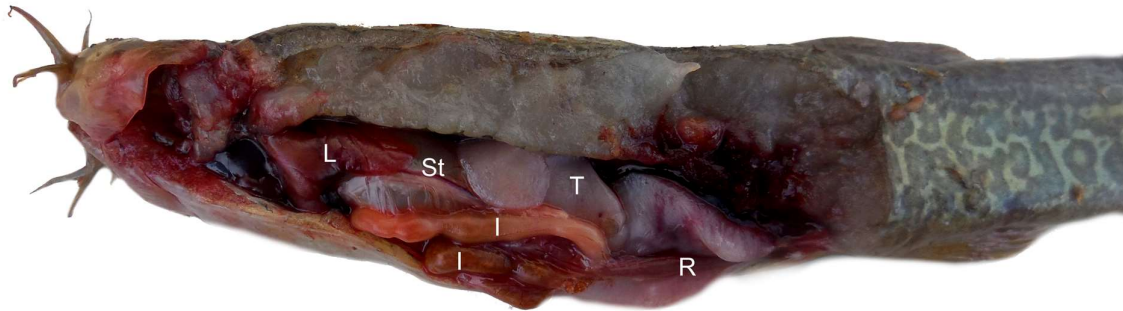


Figure 23: Left-lateral view. L = Liver, St = Stomach, T = Testes, I = Intestine, R = Rectum

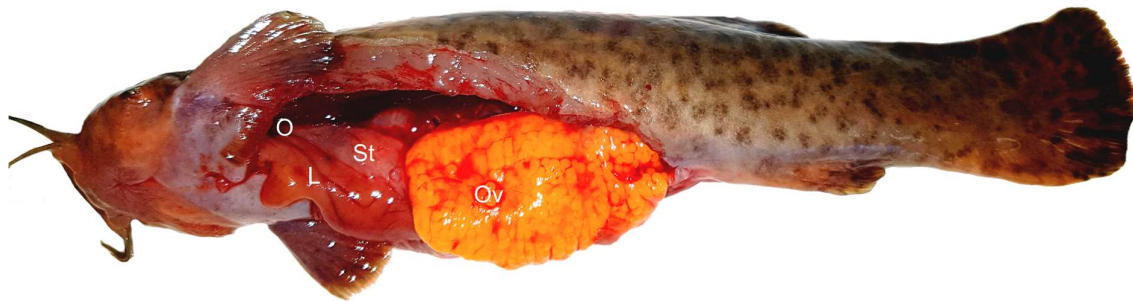


Figure 24: Left-ventrolateral view. O = Oesophagus, St = Stomach, L = Liver, Ov = Ovaries



Figure 25: Left-ventrolateral view. O = Oesophagus, K = Kidney, CS = Cardiac part of the stomach, L = Liver, PS = Pyloric part of the stomach, I = Intestine, R = Rectum



Figure 26: Left-ventrolateral view after removal of the liver. O = Oesophagus, CS = Cardiac part of the stomach, I = Intestine, R = Rectum

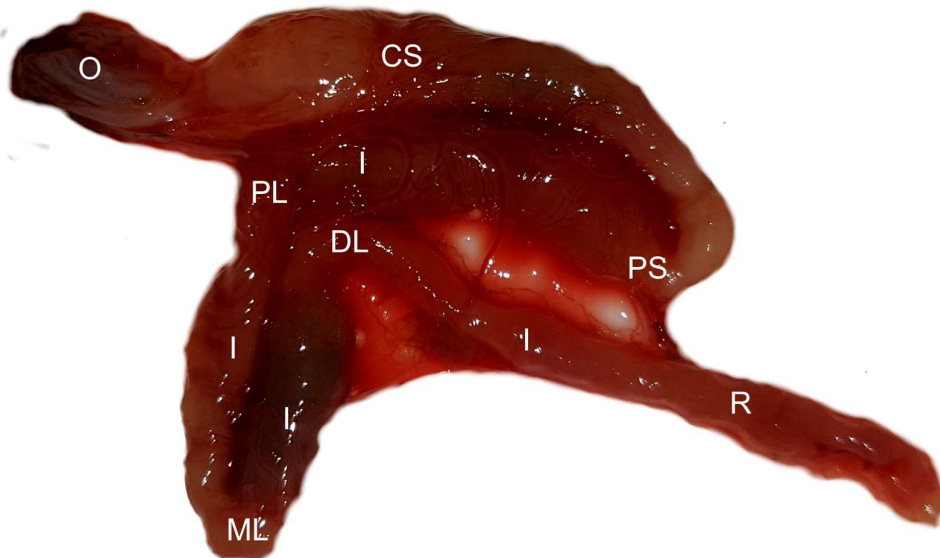


Figure 27: Left-lateral view of the alimentary tract. O = Oesophagus, CS = Cardiac part of stomach, PS = Pyloric part of stomach, I = Intestine, PL = Proximal loop, ML = Medial loop, DL = Distal loop, R = Rectum



Figure 28: Left-lateral view of the uncoiled alimentary tract. O = Oesophagus, St = Stomach, I = Intestine, R = Rectum

4.2. STATISTICAL ANALYSIS OF MORPHOMETRIC DATA

Results of the statistical data analysis can be found in Table 4. In both somatic digestive indexes, significant differences are found. The lowest ZI-index is found in *O. mykiss*, which is significantly lower than the *Orestias* species and *T. rivulatus*. *O. ispi* takes the middle position on the spectrum. The other species, *O. luteus*, *O. agassi* and *T. rivulatus*, had the highest ZI. There are no significant differences between these three species.

Some similarities are found in the comparison of the relative gut length. *O. mykiss* has the lowest RGL and *O. ispi* and *T. rivulatus* occupy the middle position. RGL of *O. luteus* and *O. agassi* is twice as large as *O. mykiss*; they respectively occupy the second-highest and highest position.

RGM is the highest in *O. mykiss* and *O. luteus*, followed by *O. agassi* and *O. ispi*; *T. rivulatus* has the lowest RGM.

Table 4: Morphometric data, digestive somatic indexes and RGM

| Parameter | <i>Oncorhynchus mykiss</i>¹ n = 21 | <i>Orestias agassi</i> n = 66 | <i>Orestias luteus</i> n = 57 | <i>Orestias ispi</i> n = 61 | <i>Trichomycterus rivulatus</i> n = 20 |
|---|---|---|---|---------------------------------------|--|
| Standard length [mm]² | 161.3 ± 37,9 | 99.4 ± 9.3 | 88.4 ± 15.6 | 53.9 ± 3.0 | 153.1 ± 21.2 |
| Body mass [g]² | 102.525 ± 74.316 | 25.360 ± 6.598 | 26.719 ± 13.525 | 2.376 ± 0.333 | 56.714 ± 23.004 |
| Gut length [mm]² | 187.8 ± 50.1 | 152.4 ± 24.9 | 151.8 ± 33.0 | 57.3 ± 4.9 | 180.8 ± 20.0 |
| Gut mass [g]² | 11.582 ± 7.595 | 1.158 ± 0.437 | 2.056 ± 1.198 | 0.119 ± 0.026 | 1.490 ± 0.617 |
| Zihler-index³ | 2.737 (2.044-3.699) _a | 5.211 (3.410-6.890) _b | 5,184 (3.902-6.481) _b | 4.302 (3.739-5.119) _c | 4.833 (3.784-6.352) _b |
| RGL³ | 0.749 (0.561-0,921) _a | 1.531 (0.952-1.980) _b | 1.711 (1.135-2.228) _c | 1.063 (0.929-1.281) _d | 1.195 (0.916-1.607) _d |
| RGM³ | 0.120 (0.054-0.198) _a | 0.064 (0.025-0.094) _b | 0.076 (0.036-0.142) _a | 0.050 (0.032-0.077) _b | 0.028 (0.018-0.054) _c |

¹In *O. mykiss*, ZI and RGL, were calculated with IL instead GL.

²Mean ± SEM

³Values are median and range between parentheses

Medians with a different subscript letter are statistically significant p < 0.05

4.3. STABLE-ISOTOPE ANALYSIS

The results of the SIA were not received before the deadline of this thesis.

4.4. STATISTICAL ANALYSIS METABOLOMICS

No significant differences were found between the native species of Lake Titicaca. It was decided to cluster the data of the native species and compare these with the non-native *O. mykiss* instead. When all data per fish species are put together without considering the type of sample, i.e. when data of all fish body sites are put together, Principal Component Analysis (PCA) reveals no apparent differences between the rainbow trout and the native species [Figure 29].

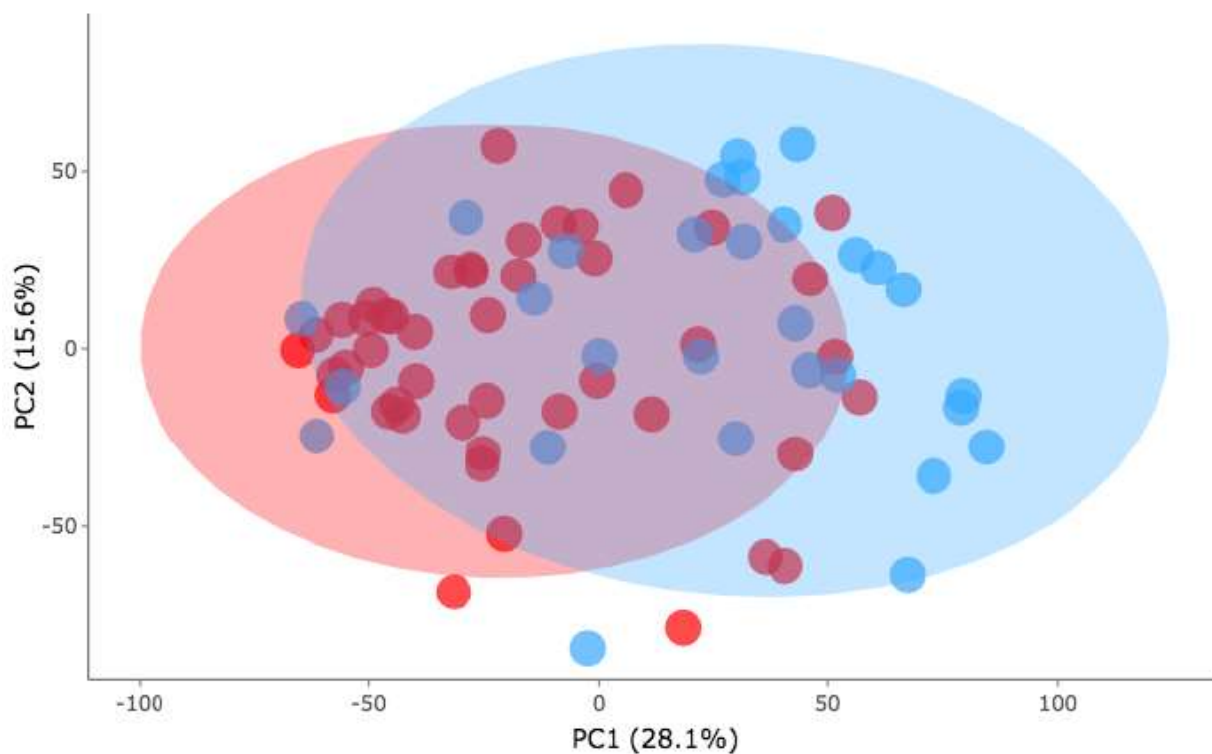


Figure 29: PCA of metabolomics data from all samples. Blue: *O. mykiss*; Red: native species; PC: principal component

However, using the Partial Least Squares Discriminant Analysis (PLS-DA) on the data instead, a clear separation of the data becomes apparent. There is a clear significant separation of the *O. mykiss* metabolomics profiles from the native species [Figure 30].

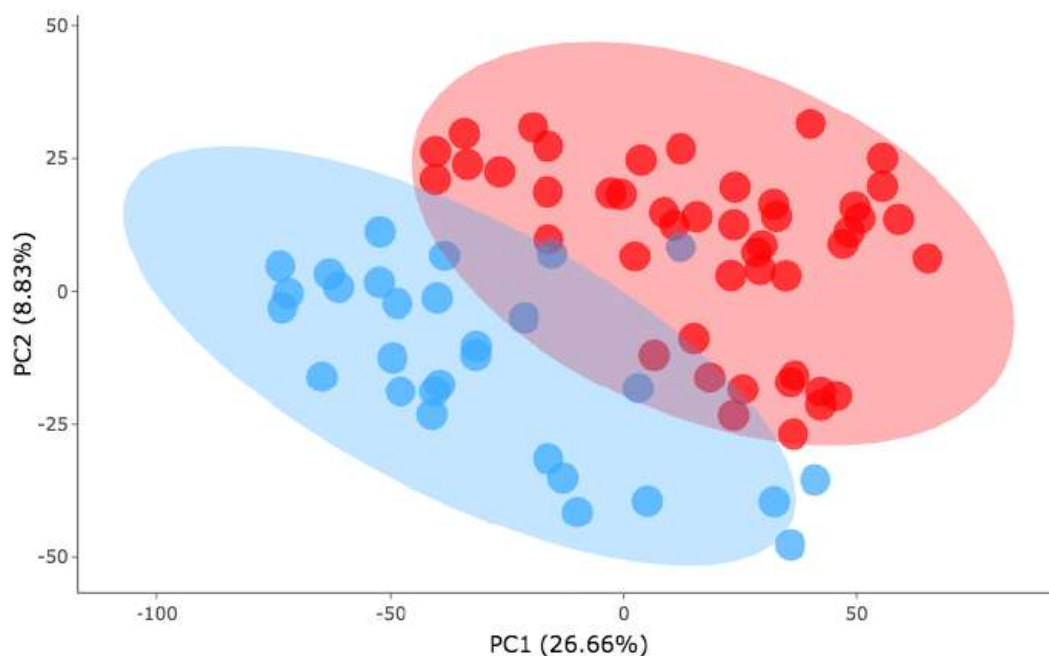
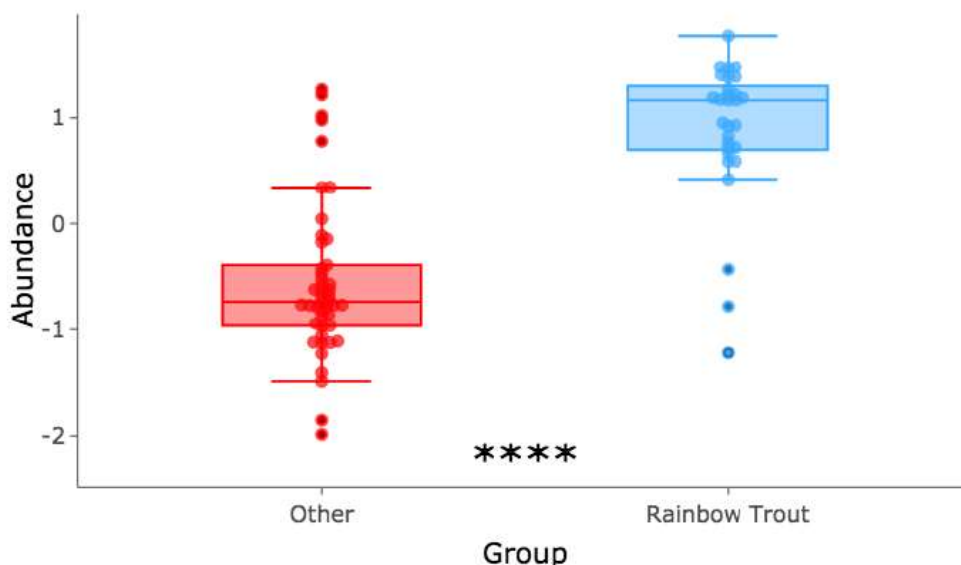


Figure 30: PLS-DA of metabolomics data from all samples. Blue: *O. mykiss*; Red: native species; PC: principal component

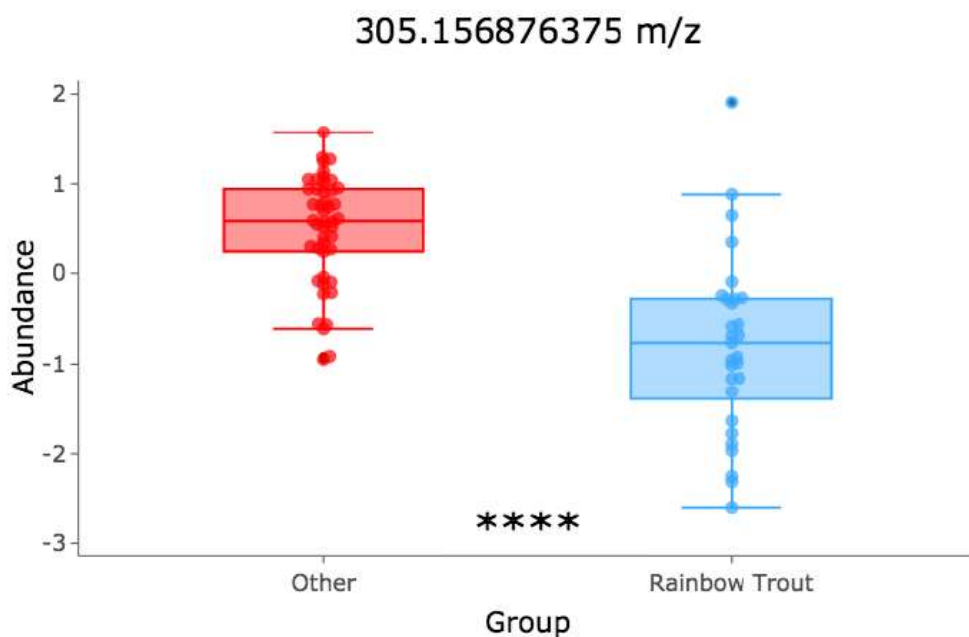
Four mass spectrometry fragments with different adducts that were related to N-acetylglucosamine, the major breakdown product of chitin by the chitinolytic system, were detected. When considering samples from all fish body sites together, the fragments did not show a consistent image. One fragment was significantly higher in rainbow trout compared with the native species [Figure 31], whereas the opposite was observed for the other three fragments [Figure 32, Figure 33 and Figure 34].

241.1291528 m/z



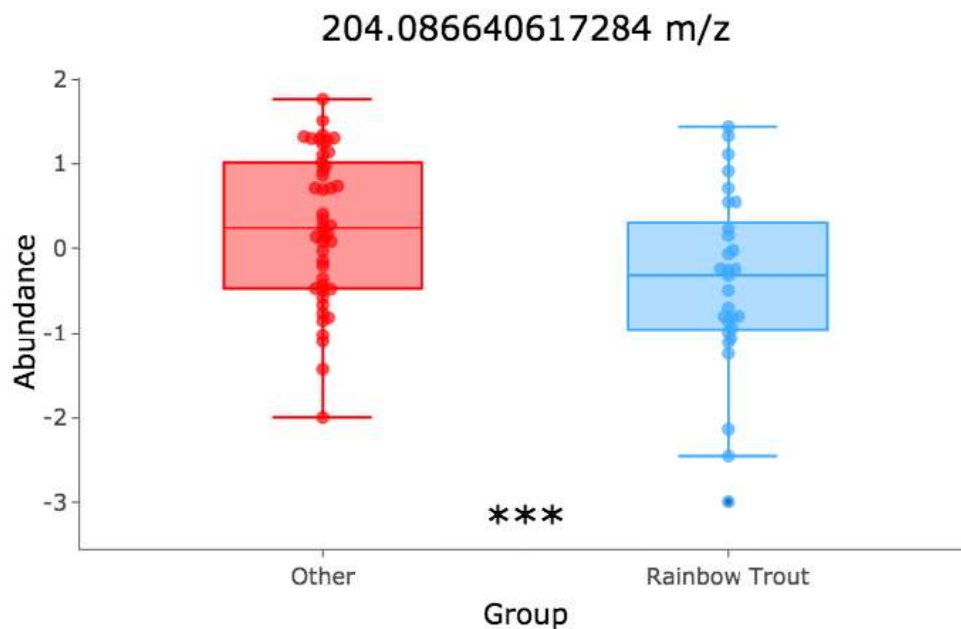
| m/z | metabolite | adduct | %isotopes | dppm |
|-------------|------------------------|------------------------------------|-----------|------|
| 241.1291528 | N-Acetyl-D-glucosamine | [M+NH ₄] ¹⁺ | 1.23 | 4.7 |

Figure 31: Boxplots showing the normalised abundance of the m/z value 241.1291528 among rainbow trout samples and samples from other species. The m/z value was found in one of the databases as the metabolite N-Acetyl-D-glucosamine with an ammonia adduct.



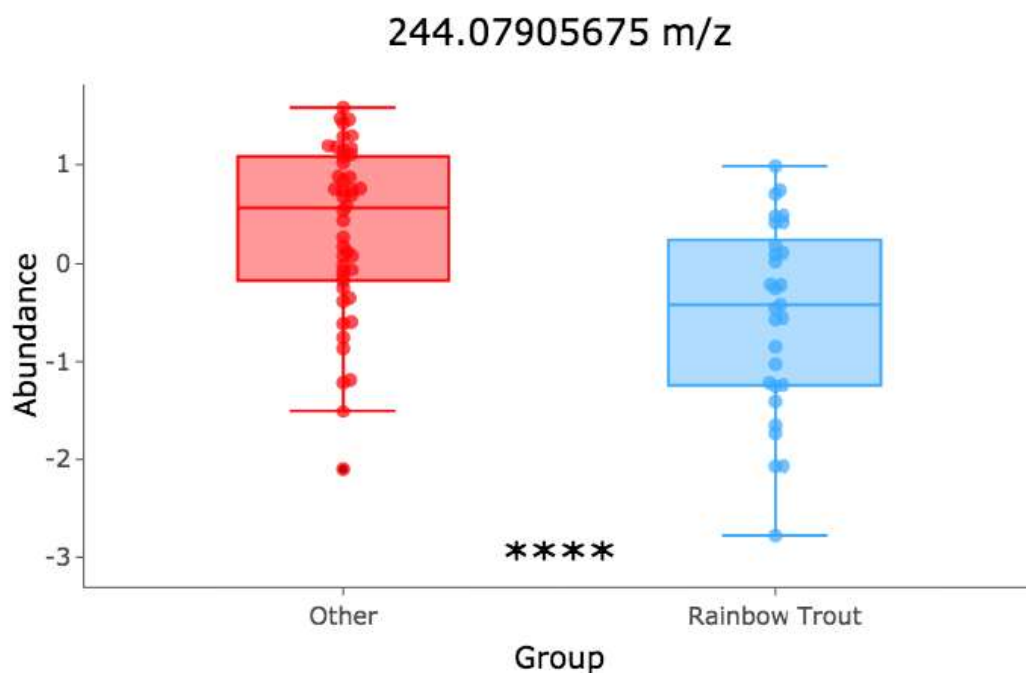
| m/z | metabolite | adduct | %isotopes | dppm |
|---------------|------------------------|--------------|-----------|------|
| 305.156876375 | N-Acetyl-D-glucosamine | [M+2ACN+H]1+ | 0.25 | 0.94 |

Figure 32: Boxplots showing the normalised abundance of the m/z value 305.156876375 among rainbow trout samples and samples from other species. The m/z value can be found in one of the databases as the metabolite N-Acetyl-D-glucosamine with an acetonitrile adduct.



| m/z | metabolite | adduct | %isotopes | dppm |
|------------------|---------------------|-------------|-----------|-------|
| 204.086640617284 | N-Acetylglucosamine | [M+H-H2O]1+ | 100 | 0.041 |

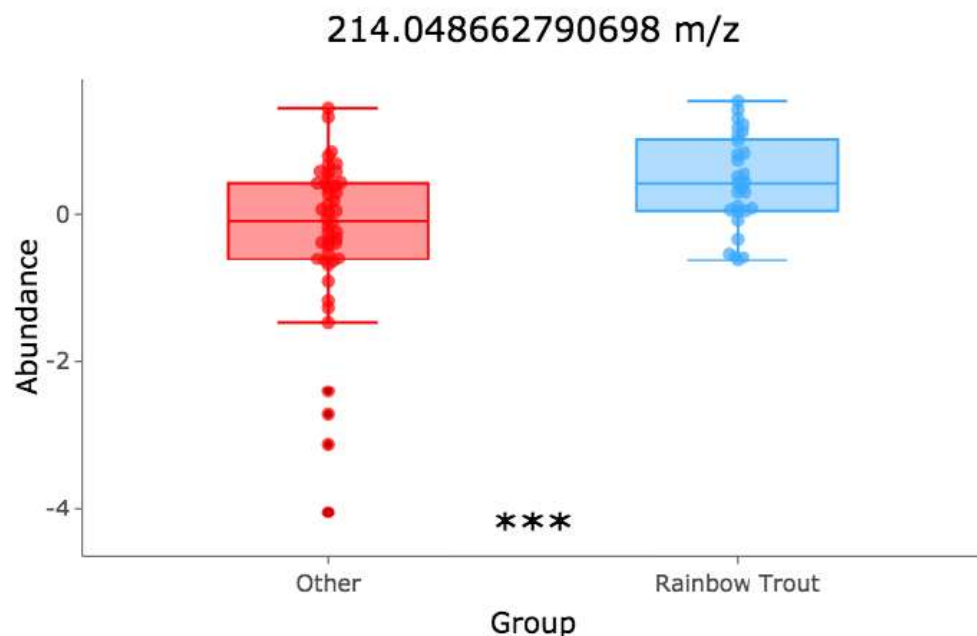
Figure 33: Boxplots showing the normalised abundance of the m/z value 204.086640617284 among rainbow trout samples and samples from other species. The m/z can be found in one of the databases as the metabolite N-Acetylglucosamine with a water adduct.



| m/z | metabolite | adduct | %isotopes | dppm |
|--------------|---------------------|----------|-----------|------|
| 244.07905675 | N-Acetylglucosamine | [M+Na]1+ | 100 | 0.42 |

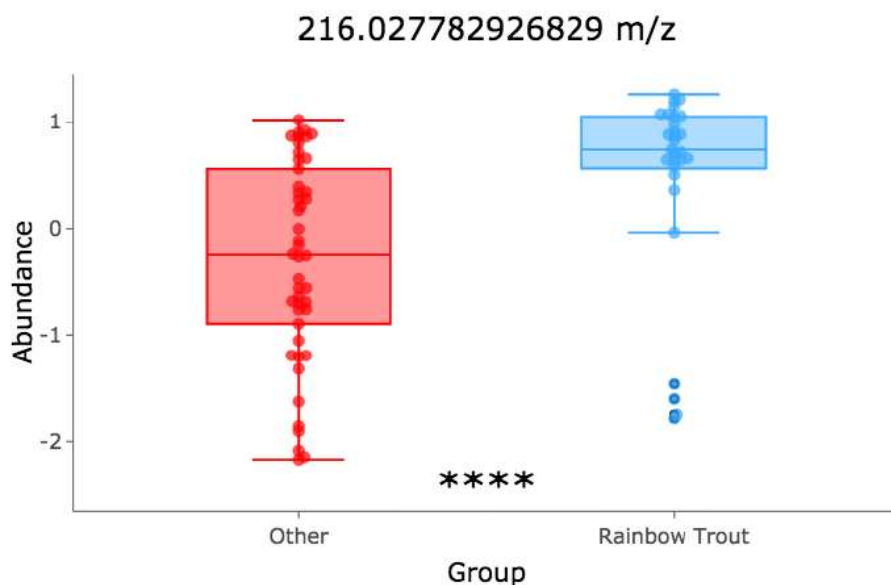
Figure 34: Boxplots showing the normalised abundance of the m/z value 244.07905675 among rainbow trout samples and samples from other species. The m/z value can be found in one of the databases as the metabolite N-Acetylglucosamine with a sodium adduct.

A further breakdown product, the deacetylated N-acetylglucosamine, appeared as two fragments with different adducts. In this case, both fragments showed higher concentrations in the native species than in rainbow trout [Figure 35 and Figure 36].



| m/z | metabolite | adduct | %isotopes | dppm |
|------------------|--------------------|----------|-----------|------|
| 214.048662790698 | beta-D-Glucosamine | [M+Cl]1- | 100 | 0.52 |

Figure 35: Boxplots showing the normalised abundance of the m/z value 214.048662790698 among rainbow trout samples and samples from other species. The m/z value can be found in one of the databases as the metabolite beta-D-glucosamine with a chlorine adduct.



| m/z | metabolite | adduct | %isotopes | dppm |
|------------------|--------------------|------------|-----------|------|
| 216.027782926829 | beta-D-Glucosamine | [M+K-2H]1- | 100 | 0.9 |

Figure 36: Boxplots showing the normalised abundance of the m/z value 216.027782926829 among rainbow trout samples and samples from other species. The m/z value can be found in one of the databases as the metabolite beta-D-glucosamine with a potassium adduct.

When looking at the blood samples only, fish species could not be separated on PCA analysis. Additionally, the PLS-DA on blood metabolome does not provide a reliable model that performs better than random. However, two fragments related to chitin could be identified, though the results appear to be contradictory. The N-acetyl-D-glucosamine fragment was higher in rainbow trout [Figure 37], whereas the N-acetyl-D-glucosamine-6-Phosphate fragment was higher in the native species [Figure 38].

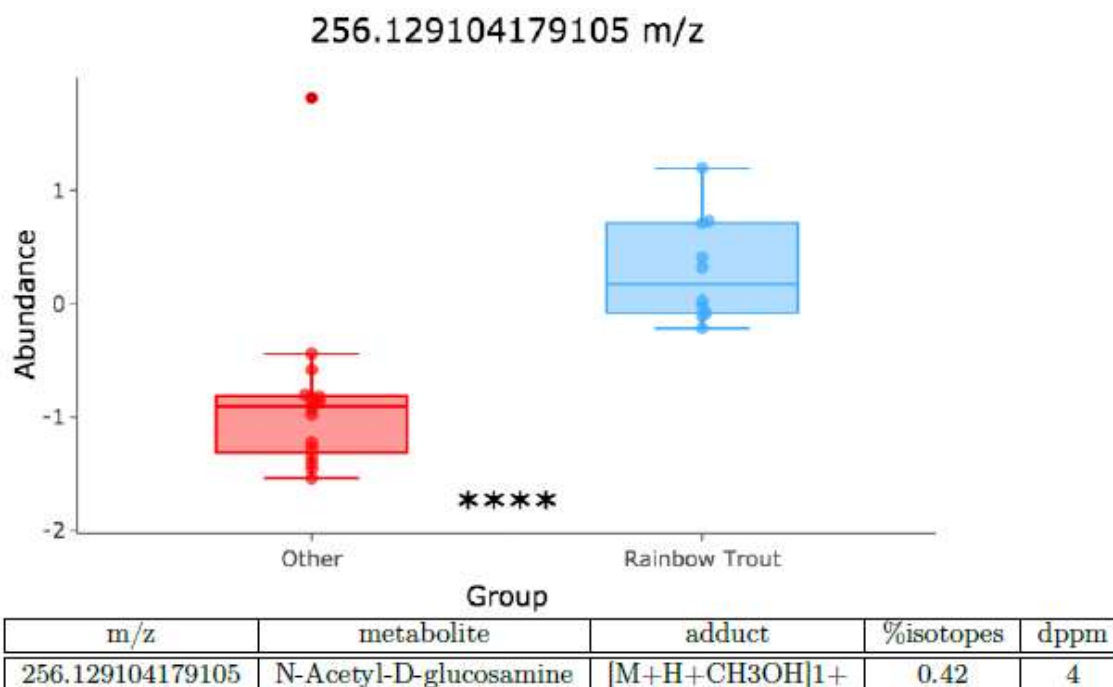


Figure 37: Boxplots showing the normalised abundance of the m/z value 256.129104179105 among rainbow trout blood samples and blood samples from other species. The m/z value can be found in one of the databases as the metabolite N-Acetyl-D-glucosamine with a methanol adduct.

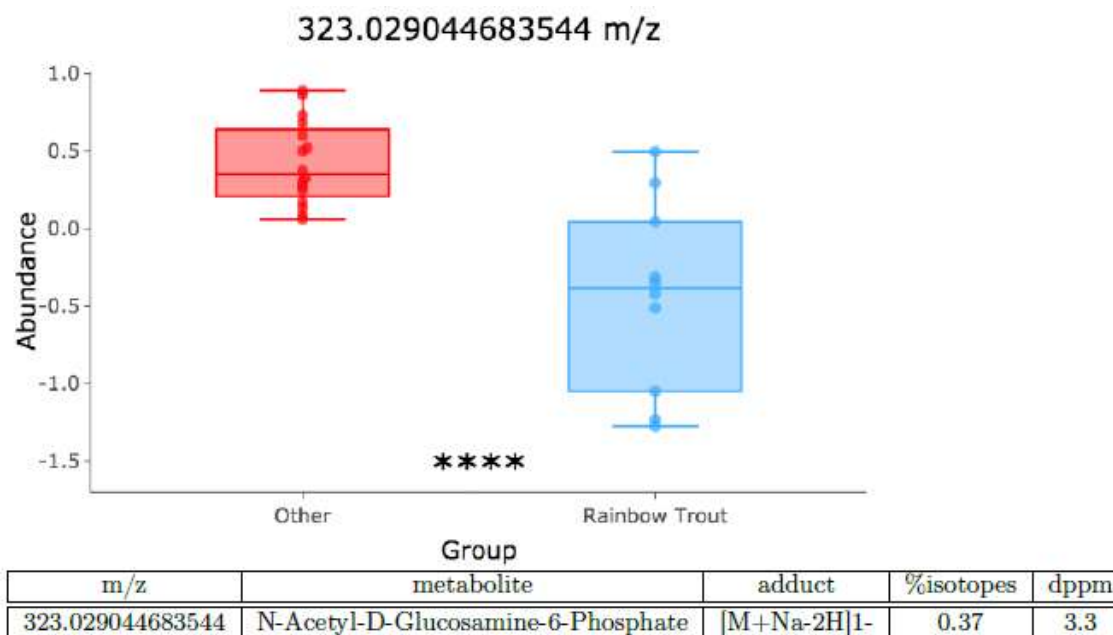
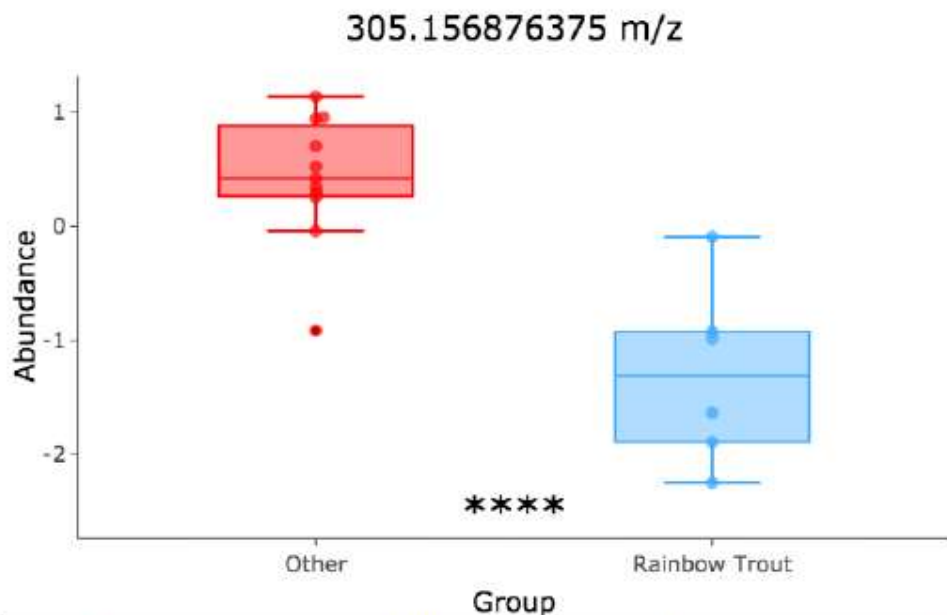


Figure 38: Boxplots showing the normalised abundance of the m/z value 323.029044683544 among rainbow trout blood samples and blood samples from other species. The m/z value can be found in one of the databases as the metabolite N-Acetyl-D-Glucosamine-6-Phosphate with a sodium adduct.

Similar to the blood results, when only the results from the proximal intestine are considered, fish species could not be separated by PCA and PLS-DA could not provide a reliable model. However, a chitin fragment, N-acetyl-D-glucosamine, is higher in the native species than rainbow trout Figure 39.



| m/z | metabolite | adduct | %isotopes | dppm |
|---------------|------------------------|--------------------------|-----------|------|
| 305.156876375 | N-Acetyl-D-glucosamine | [M+2ACN+H] ¹⁺ | 0.25 | 0.94 |

Figure 39: Boxplots showing the normalised abundance of the m/z value 305.156876375 among proximal intestinal samples of rainbow trout and from other species. The m/z value can be found in one of our databases as the metabolite N-Acetyl-D-Glucosamine with an acetonitrile adduct.

In the samples of the distal intestine, no fragments related to chitin, i.e. N-acetyl-D-glucosamine, N-Acetyl-D-Glucosamine-6-Phosphate or β -D-glucosamine, could be detected amongst the significantly different m/z values.

5. DISCUSSION

5.1. GROSS MORPHOLOGY AND MORPHOMETRY

In this study, the gut was examined from five species caught in proximity to Isla del Luna, in Lake Titicaca, a total of 225 fish were dissected; *Orestias albus* (n=66), *Orestias ispi* (n=61), *Orestias luteus* (n=57), and *Trichomycterus rivulatus* (n=20) and *Oncorhynchus mykiss* (n=22). The gross morphology of the gastro-intestinal tract was described, which previously was only available for one out of the five examined species; *O. mykiss*. It was revealed that within the examined *Orestias* spp., the most diverse and important genus by biomass of the lake, the gut morphology is very similar (Lauzanne, 1992). This research found that *Orestias* are most likely an agastric species. No apparent differences, except size, could be identified between these species. They all have an anatomically unspecialised gut. According to Lauzanne (1982), there are specific adaptations of the headgut; all known *Orestias* spp. have pharyngeal teeth which vary according to their diet. In contrast, *Trichomycterus rivulatus* did appear to be a gastric species, though their stomach was less developed when compared to the rainbow trout. Their intestine was relatively short and had three loops, but no other specialised structures. Compared to the body size and buccal cavity, the intestines had a very small diameter, which suggests that they mostly feed on smaller prey items.

Macroscopically identifiable intestinal contents indicated carnivory to various degrees. This was most obvious in rainbow trout, in which gut contents show a plethora of amphipods. Similarly, many amphipods were found in *O. agassi* and *O. luteus*, with generally more amphipods found in the intestinal tract of the former. In most *O. luteus* samples, more mollusc shells were found than amphipod skeletons, indicating that *Helioba* species are the preferred prey item. Mollusc shells are known to contain chitin, but it is fewer in content and more resilient to digestion when compared to that of exoskeletons (Poulicek and Jeuniaux, 1982). The least amount of amphipods were found in *T. rivulatus*. Additionally, in *T. rivulatus* and *O. luteus* some green, fibrous digesta was recovered, which we presume was of plant origin, indicating a degree of herbivory. These results mostly coincide with known dietary items found in these species (Monroy et al., 2014). Additional feeding items which were not found during this investigation include fish and frogs for *O. mykiss*; eggs and fish for *O. luteus*; and plant detritus for *O. agassi*. According to Vila et al. (2007), Trichomycteridae mainly feed on aquatic insects and amphipods, yet most *T. rivulatus* individuals in this study had relatively empty intestinal tracts and the few amounts of content that was present, looked more like of plant origin. In *O. ispi*, no material could be identified except fish eggs, but according to literature, this species feeds almost exclusively on zooplankton (Lauzanne, 1992). Based on these results fish can be ranked by their degrees of carnivory as follows; *O. mykiss* > *O. ispi* > *O. agassi* > *O. luteus* > *T. rivulatus*. Combined with our findings on RGM, in which the highest value was recorded for *O. mykiss* and *O. luteus* and the lowest for *T. rivulatus*, the following ranking for relative chitin quantity (which roughly corresponds with relative chitin intake) could be made; *O. mykiss* > *Orestias* spp. > *T. rivulatus*. *O. luteus* was ranked intermediate with the other *Orestias* spp. as its RGM was likely elevated due to the higher proportion of mollusc shells in its gastrointestinal tract.

When looking at the somatic digestive indexes, the lowest ZI and RGL values were recorded in *O. mykiss*. The intermediate values were recorded for *T. rivulatus* and *O. ispi*. The highest values for both were recorded in *O. agassi* and *O. luteus*. Values for *T. rivulatus* are not without scrutiny. Usually, when somatic digestive indexes are compared between gastric and agastric fish species, RGL and ZI are calculated using the intestinal length, instead of gut length. However, intestinal lengths were not measured as it was too difficult to accurately determine where the stomach ended, and oesophagus started. It might be possible that more accurate data would have placed *T. rivulatus* digestive somatic indexes between *O. mykiss* and *O. ispi*. These results coincide with the general pattern of carnivorous fish having relatively shorter guts compared to herbivorous fish, with omnivorous fish as intermediate.

5.2. METABOLOMICS

There were several limitations for the interpretation of the metabolomics data. Chitin intake was not directly measured, nor could it be detected by the metabolomics by design of the sample collection. After centrifugation of the gut contents, chitin precipitated, due to its physicochemical properties and thus blood samples should not contain any chitin. Additionally, the results of the stable isotope analysis, which could have given us more information on diet and digestion efficiency were not received on time. It can be assumed, based on dissection results, that in these samples chitin-intake of *O. mykiss* is higher than the other species. Lastly, metabolomics data will not allow us to differentiate chitinolytic activity of endogenous origin from that of exogenous origin.

In general, rainbow trout shows a different metabolite profile than the native fish species, which suggests actual differences in metabolism. However, when zooming into chitin breakdown products, results do not provide a straightforward image.

First, in the comparisons of all body sites, fragments corresponding to GlcNac, were higher in native species compared to rainbow trout, except for one. A possible explanation for this discrepancy is that the fourth fragment is not a true match with GlcNac. In DI-MS, metabolites are often detected with adducts listed as [M+X], where M is the metabolite and X is the adduct. The most common and likely adducts are the simple adducts M+/-H, +Na, and +K. Metabolites with more complex adducts can have incorrect matches. These results suggest that when considered as a whole, more end-products of complete chitin-breakdown were present in native species, even though our other findings indicated that chitin-intake was higher in rainbow trout. Thus, based on these results, it might be the case that native species are better in digesting chitin than rainbow trout. This is remarkable because substantial levels of chitinase and NAGase have been recorded in rainbow trout: this indicates that levels in the native species are possibly even higher (Okutani et al., 1967a; Micha et al., 1973). However, the glucosamine levels, when all body sites were considered showed the reverse pattern. Glucosamine is deacetylated GlcNac. Therefore, it might be concluded that in rainbow trout, there is a higher deacetylase activity or more efficient metabolisation of GlcNac into glucosamine.

When separate body sites were compared between *O. mykiss* and the native species, the following observations could be made. In the proximal intestine, a fragment which was identified as, chitin's monomer; GlcNac, was found more often in native species than rainbow trout. This implies higher chitinolytic activity in the native species or more efficient GlcNac absorption by trout. However, in blood samples, GlcNac was more abundant in rainbow trout than endemic fish. Yet, GlcNac 6-phosphate showed the opposite pattern. This discrepancy in data can be clarified by two reasonings. On the one hand, absorptive processes in rainbow trout are more efficient in GlcNac than native fish, which is why more GlcNac is found in the blood of the former, despite the latter having a higher quantity in the intestinal lumen. On the other hand, this may be the consequence of a difference in metabolic pathways between the fish. According to Krogdahl et al. (2005), GlcNac is either directly used in a synthetic pathway requiring GlcNac & glucosamine or funnelled into glycolytic intermediates. The contradictory results between chitin metabolites in blood between both groups could be the consequence of rainbow trout preferably metabolising GlcNac directly, whereas in the native species GlcNac is preferably used to form glycolytic intermediates. This is supported in part by the data of all fish body sites, whereby higher concentrations were found for glucosamine in *O. mykiss*.

Lastly, metabolomics analysis found no significantly different chitin-related metabolites between the fish species in the distal intestine. It is assumed that by then most of the chitin metabolites have been absorbed by the fish.

In conclusion, based on results from all fish body sites, chitinolytic activity is higher in native species; however, absorption and metabolisation are likely more efficient in rainbow trout.

5.3. CONCLUSION AND FUTURE PERSPECTIVES

Our data indicate that the examined native fish species of Lake Titicaca likely have higher chitinolytic activity than rainbow trout. Nevertheless, the native species are likely consuming less chitin than rainbow trout. Results by Gutowska et al. (2004) indicate that there is a negative correlation between gut length and chitinase activity. According to our results, chitinolytic activity was lower in rainbow trout, which had the relatively shortest intestinal tract. However, this difference may be explained by the difference in phylogeny between the two groups. Future studies could use more precise techniques, such as chitinase assays or gene expression to measure chitinase activity between *Orestias* species, to see if there is a relationship between gastrointestinal length and chitinase activity. The added benefit of measuring gene expression is that it would allow differentiating between the contributions by exogenous (i.e. of prey or bacterial origin) and endogenous enzymes to total chitinolytic activity.

The correlation between chitinase activity and mechanical processing of food items is corroborated by our results. Amphipod skeletons and mollusc shells are better processed by the teeth and a more anatomically specialised gastrointestinal tract (i.e. gastric acid and enzymes, stronger muscular contractions) in rainbow trout than by the microscopic pharyngeal teeth and stomachless alimentary tract in *Orestias*. However, our data from metabolomics from all fish body sites indicate that chitinolytic activity is higher in *Orestias* spp. than *O. mykiss*.

Our results indicate that absorption and metabolisation of the end-products are more efficient in rainbow trout, even though it has been previously reported in the literature that chitin digestion in rainbow trout is poor. The metabolomics data across all species indicate that chitin is used at least partially as a source of nutrients and energy. Still, data of the gut contents found that most of the chitinous exoskeleton and chitin-containing mollusc shells is undigested. Data of the stable isotope analysis, which will be obtained in the near future, will hopefully shed more clarity on the digestive efficiency and metabolisation of prey in these species.

Undigested exoskeletons and/or mollusc shells are more likely to cause intestinal blockage in native species, due to the relatively smaller diameter of the alimentary tract and presence of multiple intestinal loops. Intestinal obstruction is less expected to occur in *O. mykiss*, which has a relatively straight intestine. Therefore, we assume that chemical disruption by chitinases is much more important in native species. More research will be needed to confirm the importance of chitinous material in the nutrition of wild fish as well as their application as replacement of fish meal for captive fish in aquaculture systems.

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