# Studies on the feeding physiology of the hydrothermal vent crab *Xenograpsus testudinatus* (Decapoda, Brachyura)

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**Abbreviations**

| | |
|---|---|
| ALT | Accessory lateral teeth |
| ANOVA | Analysis of variance |
| BSA | Bovine serum albumine |
| CS | Cardiac stomach |
| CHYM | Chymotrypsin |
| DCM | Dichlormethane metanol |
| EPA | Eicosapentaenoic acid |
| DHA | Docosahexanoic acid |
| EDTA | Ethylenediaminetetraacetic acid |
| FA | Fatty acid |
| FID | Flame ionisation detector |
| FFA | Free fatty acids |
| GC | Gas chromatograph |
| LT | Lateral teeth |
| MED | Median tooth |
| MUFA | Monounsaturated fatty acids |
| BAPNA | N-Benzol-L-arginine-4-nitroanilide-hydrochloride |
| SAAPNA | N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide |
| P4G5 | 4% paraformaldehyde with 5% glutaraldehyde |
| PB | Phosphatebuffer |
| PL | Polarlipids |
| PUFA | Polyunsaturated fatty acids |
| PO | Prepectineal ossicle |
| ROC | Routine oxygen consumption |
| SFA | Saturated fatty acid |
| SEM | Scanning Electron Microscope |
| SDS | Sodium dodecyl sulphate |
| ST | Sterol |
| SE | Sterolester |
| TL | Total lipid |
| TLC | Thin layer chromatography |
| TCA | Trichloroacetic acid |
| TAG | Triacyglycerol |
| TRY | Trypsin |
| UO | Urocardiac ossicle |
| WE | Waxester |
| ZO | Zygocardiac ossicle |

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**Abstract**

This work investigated the feeding physiology of the hydrothermal vent crab *Xenograpsus testudinatus*, (Crustacea: Brachyura). This species is frequent near Kueishan Island (Taiwan) where it lives in shallow waters close to the hydrothermal vents located in this area. *X. testudinatus* is an outstanding crustacean, which is adapted to sulphur-rich, and thus, potentially toxic environment. This species is an integral member of the thermal vent communities and food webs at Turtle Island. It established a specialized feeding behaviour mainly on dead zooplankton organisms, which were killed by the toxic discharges from the vents and fell to ground. Just during slack water, when there is almost no or just little current, the crabs leave their cavities for feeding on this "marine snow".

This study investigated the energetic demands of the cabs in terms of oxygen requirements, showing routine respiration of $3.515 \pm 0.946$ [$\mu mol \cdot g_{FM}^{-1} \cdot h^{-1}$] at 25°C and daily energy demands of 326 J. The physiological properties to utilize the prey in terms of digestive capacities, and the characterization of major digestion enzymes showed high activities for proteolytic enzymes, which were comparable to those of the Antarctic krill *Euphausia superba*. This feature can be regarded as an adaptation to irregular and patchy food availability. Furthermore, enzymes showed high stabilities in order to cope with elevated temperatures and inorganic inhibitors like $Cu^{2+}$, $Fe^{2+}$ or $Co^{2+}$. This feature can be regarded as essential to function in an extreme Habitat over long exposure times. The alkaline pH optimum and wide pH stability (pH 6-10) for trypsin and chymotrypsin, suggesting an alkaline internal pH, may be interpreted as a detoxification mechanism, favouring the less toxic $HS^-$ over the more toxic $H_2S$. Lipid analysis demonstrated the potential of this crab to store significant amounts of lipid (50-60% of mid gut gland DM) and their utilization during starvation. This feature can be seen as a physiological answer to prolonged starvation periods, caused by local currents and tides, making food not available. The lipid reserves are mainly stored as triacylglycerides (TAG). The midgut gland fatty acid composition shows high amounts of MUFAs (mainly 16:0; 16:1(*n*-7); 18:1(*n*-9) and 18:1(*n*-7)). The occurrence of 18:1(*n*-9) in high numbers indicates carnivory. Furthermore, the presence of 16:1(*n*-7) and 18:1(*n*-7) trophic markers suggests a diet containing bacteria, due to the fact that 16:1(*n*-7) and 18:1(*n*-7) producing algae or phytoplankton are rare in this habitat. These findings reflect physiological properties and energetic strategies, which enables this hydrothermal vent crab to survive in this extreme habitat.

**Zusammenfassung**

Diese Arbeit untersucht die Ernährungsphysiologie des Krebses *X. testudinatus* (Brachyura), heimisch an Flachwasser-hydrothermalquellen vor der taiwanesischen Ostküste bei der Kueishan Insel. Dieser Krebs ist in der Lage in einem schwefelreichem, und somit extrem toxischem Habitat zu überleben. Um in diesem lebensfeindlichen Habitat ausreichend Nahrung zu bekommen, hat dieser Krebs eine sehr aussergewöhnliche Ernärungsstrategie entwickelt. Er ernährt sich hauptsächlich von Zooplankton, das durch die toxischen Schwaden der Quellen getötet wird und zu Boden sinkt. Da diese Nahrungsquelle stark Wetter und Gezeiten abhängig ist, ist Nahrung nur sporadisch, aber wenn, in großen Mengen verfügbar. Nur zu diesen Zeitpunkten verlassen die Tiere ihre Verstecke und schwärmen aus, um so viel wie möglich von dem „Marine snow" zu erbeuten.

Metabolische Raten dieses Krebses zeigten einen Sauerstoffverbrauch von $3.515 \pm 0.946$ [$\mu mol \cdot g_{FM}^{-1} \cdot h^{-1}$] bei 25°C und somit einen Tagesenergiebedarf von etwa 326 J. Untersuchungen der Enzyme der Mitteldarmdrüse haben gezeigt, dass diese Art sehr aktive Proteasen besitzt, die in ihren Aktivitäten denen des antarktischen Krill ähnlich sind. Diese Fähigkeit kann als Anpassung an eine Nahrungsquelle gesehen werden, die nur sporadisch auftritt, aber dafür große Mengen an Nahrung liefert. Ferner konnte gezeigt werden, dass die proteolytischen Enzyme sehr stabil sind. Sie besitzen erhöhte Toleranzen gegen Temperatur und anorganische Inhibitoren, was als Notwendigkeit angesehen werden kann, in einem extrem reduzierenden Habitat langfristig zu bestehen. Das stark alkaline pH Optimum und die weite pH Stabilität (pH 6-10) der Serinproteasen Trypsin und Chymotrypsin weisen auf interne Detoxifizierungsmechanismen hin, die bei alkalischen Bedingungen das Gleichgewicht von $H_2S$ zum weniger toxischen $HS^-$ verschieben. Die Fähigkeit große Mengen an Lipid (50-60% der Mitteldarmdrüsentrockenmasse), haupsächlich als TAGs, in der Mitteldarmdrüse zu speichern ermöglicht den Tieren lange Hungerphasen zu überdauern. Diese Fähigkeit kann als physiologische Antwort auf eine Nahrungsquelle gesehen werden, deren Verfügbarkeit von Gezeiten und lokalen Strömungen abhängig ist.  Die Fettsäuremuster dieser Tiere zeigt große Mengen an einfach ungesättigten Fettsäuren (hauptsächlich 16:0; 16:1($n$-7); 18:1($n$-9) und 18:1($n$-7)). Große Mengen an 18:1($n$-9) deuten auf eine carnivore Ernährung hin. Ferner zeigen hohe Konzentrationen von einfach ungesättigten Fettsäuren der ($n$-7) Serie eine mögliche, ergänzende bakterivore Ernährung. Die Ergebnisse dieser Arbeit zeigen physiologische Eigenschaften, die es diesem Krebs ermöglichen, in diesem extremen Habitat zu überleben.

**Figures and Tables**

## 1. Introduction

### *1.1. Hydrothermal vents*

Hydrothermal vents are fissures in the planets surface where geothermically heated water issues from. Hot vents are often found in areas of volcanic activity like divergent tectonic plates, ocean basins and hotspots. The discharges from these underwater hydrothermal vents mainly consist of seawater that enters into the cracks and porous sediments and becomes heated by the upwelling magma. Sometimes cylindrical chimney structures can be observed where the hot water emerges from the seafloor. These chimneys consist of deposits of anhydrides. Additionally, sulphides of iron, copper and zinc accumulate in the chimneys gaps making the chimney more stable over the time.

Hydrothermal vents found in the deep sea, usually on mid ocean ridges form oases of life in an environment that is poor in biomass. These ecosystems depend on chemoautotrophic bacteria which represent the basis of the food chain of these communities. Higher organisms found near hydrothermal vents demand special physiological features to survive in such an inhospitable environment.

One striking characteristics of hydrothermal vents is the enormous variation in water temperature. The temperature can range from 2°C to 400°C within a short distance of one centimeter and animals may have occasional brief contact with 100°C hot water (McMullin et al. 2000). Temperature directly affects various biological systems of organisms such as enzymes or membrane lipid bilayers. High temperatures can increase reaction rates and affect reaction equilibria. Additionally high temperatures can cause protein denaturation resulting in a complete loss of function (Somero and Suarez 2005).

Vent and seep fluids are highly reduced and contain significant levels of sulphide. Oxygen concentrations in vent habitats vary inversely with temperature and organisms in areas of actively mixing hydrothermal and ambient water may experience rapid fluctuations in both, temperature and oxygen (Childress 1995). Many organisms found in hypoxic environments are able to maintain aerobic respiration and normal metabolic rate even at very low oxygen tensions. Some vent fauna, such as the crab *Bythograea thermydron,* have been shown to oxyregulate to very low levels of environmental oxygen (Mickel and Childress 1982). Discharges from hydrothermal vents contain high amounts of toxic compounds like heavy metals or sulphides. The latter might be the most abundant and well studied compound released by hydrothermal vents that can

strongly influence biological systems. Exposure and the impact of hydrogen sulphide on marine invertebrates received considerable attention since the discovery of deep sea hydrothermal vents. It was shown that hydrogen sulphide can serve as energy source for chemoautotrophic bacteria (Van Dover and Lutz 2004; Kalanetra et al. 2005) and thus, being one of the central factors for the formation of animal communities in hydrothermal areas. On the other hand hydrogen sulphide is an extremely toxic compound to aerobic organisms, mainly due to its ability to inhibit cytochrome-c oxidase, a central enzyme in the electron transport chain of mitochondria. Moreover, it is well established that sulphides, as well as heavy metals are able to inhibit enzymes activities (Nicholls and Hildebrandt 1978; Hill et al. 1984; Lopez-Lopez et al. 2003).

Sulphides and heavy metals may be the major factors making deep sea as well as shallow water hydrothermal vents inhospitable for metazoans. Exclusion of these compounds from sensitive tissues and oxidation within the body are the two best documented strategies to prevent poisoning among vent and seep animals (Powell and Somero 1983). Exclusion of sulphide and other toxic compounds from tissues may involve physical, biological, or chemical barriers around or within an organism. Thick tubes or cuticles may reduce or prevent exposure of some external tissues to sulphide. Epibiotic bacteria and abundant metal ions may oxidize sulfide before it gets in contact with external tissues (McMullin et al. 2000; Girguis et al. 2002). Another very direct and efficient method to avoid sulphide poisoning is to shift the internal pH to alkaline (pH >7) which favours the formation of the less toxic $HS^-$ over the more toxic $H_2S$ (Goffredi et al. 1997). What may separate vent and seep species from other marine organisms is not the detoxification mechanism, per se, but rather the ability of to function effectively at high metal concentrations and over long exposure times (McMullin et al. 2000).

### 1.2. Xenograpsus testudinatus *at Kueishan Island*

Fife volcanic Islands rise up along the axis of the Okinawa Through back-arc basin that extends from the north-east of Taiwan to Unzen volcano in Kyushu. The south-westernmost island is Kueishan Dao (Turtle Mountain Island, Fig. 1) located 9.1 Km eastward from the nearest fishing harbour Wu-Shi off Taiwan. Kueishan Island with a maximum height of 401 m above sea level and an area of 2.85 $Km^2$ consists of andestic lava flows and volcanoclastic material. The geological era of the major volcanic activity on Turtle Island is considered to be the Pleistocene although this area still displays active fumaroles and solfataras. The narrow 200m-wide Kueishan

island shelf drops off abruptly to 50m and the vents are located on the eastern edge with 80% of them at depth of 8 to 20 m. All off them spew sulphur-rich plumes with temperatures of up to 65-112°C and a low pH of 1.9-4.6. (Jeng et al. 2004). There used to be several large chimneys, up to 6 m high discharging large plumes into the surrounding seawater, but after the heavy earthquake in 2003 most of these large chimneys collapsed and were mainly buried under rocks. The sulphurous plumes of the hot vents usually flow north-east, east or south-eastward along the local currents. Consequently coral reefs are only present at the western edge of the island where the toxic water currents do not reach. Until 1977 Kueishan Island was under strict military control and access was prohibited. Although fishermen often reported that there was volcanic activity and a strong smell of sulphur in this area access for scientific investigations was denied. First in 1977 when the island was out of use as military training zone scientist were able to examine the islands biodiversity and the shallow water hydrothermal vents.

The hydrothermal vent crab *Xenograpsus testudinatus* inhabits endemically this unique environment. This area offers several shallow-water hydrothermal vents in depths between 30 to 50 m (Jeng et al. 2004). The vents are characterised by low pH (1.8 - 4.8) and sulphur-rich discharges which reach temperatures of 65 to 116°C. Moreover, the vents release various gases mainly carbon dioxide, nitrogen, oxygen, sulphur dioxide, and hydrogen sulphide (Jeng et al. 2004). The crabs live close to the hydrothermal vents and, thus, are adapted to the conditions which are otherwise hostile against life. The waters around the hydrothermal vents are poor in nutrients. However, the crabs established an opportunistic and specialized feeding behaviour mainly preying upon dead zooplankton organisms which were killed by the toxic discharges from the vents and fell to ground. Just during slack water, when there is almost no or just little current, the crabs leave their crevices for feeding rapidly on this "marine snow" (Jeng et al. 2004). Accordingly, the crabs are exposed to toxins in two different ways: on the one hand they are exposed to the surrounding toxic water, and on the other hand they ingest prey which has previously been killed by the toxins in the water. Accordingly, the question arises how *X. testudinatus* is capable of coping with these unfavourable conditions and which kind of adaptations it developed to do so.
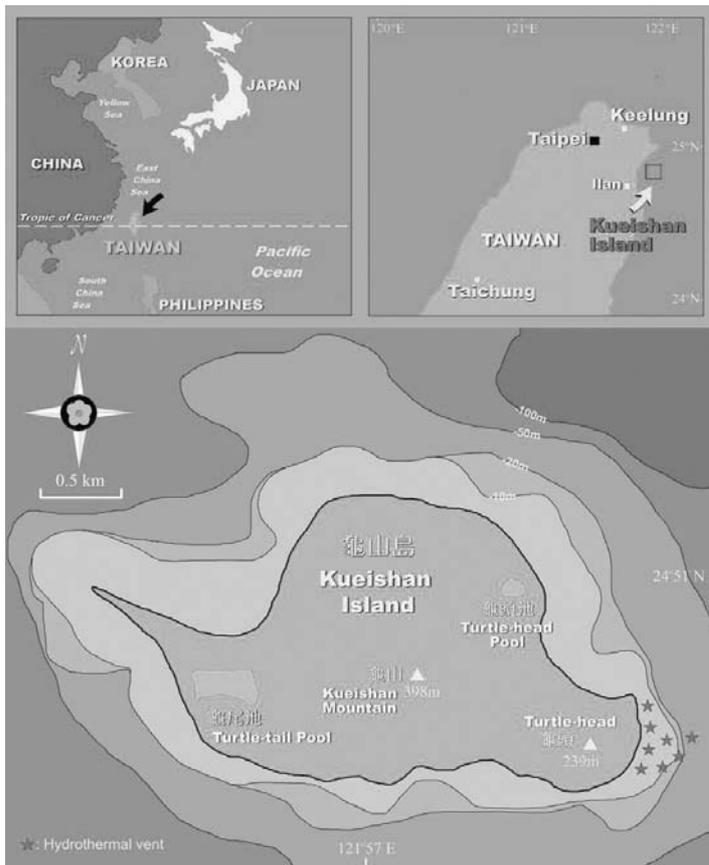
Fig. 1. Map of Kueishan island, indicating its location off Taiwan´s east coast. and its size of 3.1 Km in length. Kueishan island, meaning "Turtle Mountain Island" received its name because of the turtle-like shape of the island. The distance from the western to the eastern tip of the island covers 3.1 km. The hydrothermal venting area is located at the east end of the island which forms the "head of the turtle".

## 1.3. Metabolic rates and energy acquisition

The study on routine oxygen consumption provides significant information about the metabolic, energetic and nutritional properties of an organism. Respiration rates are influenced by many factors of which temperature can be considered as one of the most important (McMullin et al. 2000; Robertson et al. 2002). According to all biological processes the increase of temperature will lead to elevated rates until a certain temperature maximum is reached where denaturation of proteins initiates. The characteristic temperature dependent change of rate of a biological system can be expressed by the $Q_{10}$ temperature coefficient, which is calculated according to the van t`Hoff`s law:

$$Q_{10} = \left( \frac{R_2}{R_1} \right)^{10/(T_2 - T_1)}$$

With $R_1$ and $R_2$ representing the oxygen consumption at the temperatures $T_1$ and $T_2$.

The respiration curve and $Q_{10}$ values can tell the range of temperature to which the animal adapted. For example, the respiration rates of tropical species are compared to those of temperate or polar species at natural ambient temperatures. When exposed to tropical temperatures, respiration of polar species does not rise according to the $Q_{10}$ rule (Kunzmann et al. 2007), indicating that this organism is not adapted to the certain tempearature range, vice versa. In deep sea organisms metabolic rates were considered to be low in general, due to environmental characterisics like low temperatures and food scarcity. If these deep sea characteristics cause depression of metabolic rates, those shoud be high at nutrient rich hydrothermal vents. However, recent studies on respiration and growth rates in some deep-sea hydrothermal vent organisms suggest that metabolic rates correlate rather with the degree of mobility than with any environmental factors (Smith and Teal 1973; Van Dover and Lutz 2004). Such assumptions were manifested by studies on metabolic rates for vent and near-vent zooplankton (approximately 0.7 to 0.74 $\mu mol \cdot g_{FW}^{-1} \cdot h^{-1}$ at 1.68 °C) (Smith Jr. 1985) which were lower than those observed for copepods species collected from benthic boundary layers at 2000 m off the coast of Southern California (1.6 $\mu mol \cdot g_{FW}^{-1} \cdot h^{-1}$ at 3 °C) (Childress et al. 1989). Furthermore, Mickel and Childress (1982) determined oxygen consumption for the vent crab *Bythograea thermydron* (Fig. 5) and found them to be similar to non-vent deep sea crustaceans with an equal degree of mobility (Mickel and Childress 1982).

From a nutritional point of view oxygen consumption rates additionally provide information about a general energetic demands of the crab. Under aerobic conditions the consumption of oxygen is a suitable indirect calorimetric measure to estimate the energy demand in crustaceans by applying a given respiratory coefficient (RQ). The respiratory coefficient is calculated from the ratio [$CO_2$ produced / $O_2$ consumed]. Using a RQ of 0.85 will allow for the calculation of carbon demand (energy) assuming that the crab does not metabolize pure fat or pure carbohydrates but a mixed diet. The energy requirements, in turn, correspond with the amount of food to be consumed by the crabs.

*1.4. Digestive process in decapod crustaceans*

The feeding process of decapod crustaceans can be separated into three general phases. The uptake of food that is facilitated by the mouthparts, especially the mandibles, can be considered as the first step of food ingestion and mastification. The second step of food utilization takes

place in the cardiac stomach of the crustacean. Here, the ingested food items are mechanically disrupted by the gastric mill and most nutrients are cleaved by hydrolytic enzymes. The cardiac stomach is connected with the mid gut gland, the place where the last step of nutrient is located. The chyme with digested food compounds is pressed into the filamentous tubules of the mid gut gland and becomes absorbed by the epidermal cells.

### 2.4.2. Digestive enzymes

Besides the mechanical disruption of ingested food by the mouthparts or the gastric mill the enzymatic cleavage of food is essential to hydrolyse large polymers into small compounds which then can be absorbed by the midgut gland cells. Three major groups of digestive enzymes can be defined of which the proteolytic enzymes facilitate the digestion of proteins, the lipases and esterases the digestion of lipids and glycanases hydrolyse carbohydrates.

Proteases: The presence of proteolytic enzymes in the digestive system of crustaceans has been well investigated (Tsai et al. 1991; Oh et al. 2000; Rudenskaya et al. 2000; Teschke and Saborowski 2005). Trypsin, an endopeptidase, was found in a variety of crustaceans. Trypsins have a pH optimum between pH 7 and 9 and a molecular weight of about 20,000 to 25,000 kDa (Rudenskaya et al. 2004). Trypsins specifically hydrolyse peptide bounds arginine and lysine residues at their carboxyl side. Other well studied proteolytic enzymes are the chymotrypin like proteases, which were often reported to show collagenolytic activity.

Lipase/Esterase: The lipolytic enzymes of crustaceans have so far received less attention than the proteolytic enzymes, although lipid metabolism was shown to be essential in many crustaceans groups. Lipase and esterase cleave ester bonds of triacylglycerides or diacylglycerides and release free fatty acids which are then available for further metabolic steps.

Glycosidases: These enzymes catalyze the hydrolysis of glycosidic bonds, generating two smaller sugars. Their occurrence in crustaceans is well documented especially in those who digest plant material containing cellulose or hemicellulose. Furthermore glycosidases like lysozyme or N-acety-ß- glucosaminidase are capable of degrading bacterial cell wall structures that displays an important role for the utilization of bacteria as a food source or bacterial defence.

*1.4.1. Digestion enzymes in decapod crustaceans (characteristics, features etc)*

As decapod crustaceans represent a very diverse group of animals which have conquered a great variety of habitats, these animals are frequently subject of enzymological studies (Tsai et al. 1991; Rudenskaya et al. 2000; Sakharov and Prieto 2000; Lopez-Lopez et al. 2003; Saborowski et al. 2004). Particularly digestive enzymes evoked larger interests since they form the functional link between the metabolic requirements of an organism and its nutritive environment and its dietary habits, respectively. Studies on the digestive enzymes revealed information about the digestive potential of decapod crustaceans and were consequently extensively pursued in aquaculture research.

The activities and characteristics of the digestive enzymes produced by an organism provide information about its digestive capacities on the one hand, and on the other hand, may be indicative for the types of food that is preferably utilized (Klinger et al. 1997). The characterization of gastric enzymes has been successfully applied many times in order to illustrate aspects of the feeding physiology of crustaceans from different habitats (Saborowski and Buchholz 1999; Lopez-Lopez et al. 2003; Wilde et al. 2004; Knotz et al. 2006). Studies have clearly demonstrated that digestion enzymes reflect typical feeding behaviours of herbivorous, omnivorous and carnivorous crabs. Herbivore species generally show higher cellulase and amylase activity, whereas carnivorous species express highly active proteolytic enzymes like trypsin or chymotrypsin (Johnston and Freeman 2005). However, the distribution of enzymes between the extra-cellular- and intra-cellular compartments of the midgut glands provides important information about the digestive strategies (Saborowski et al. 2006). Serine-proteinases appear in the extra-cellular compartment and facilitate already in the stomach the first step in protein hydrolysis. In contrast, cysteine proteinases predominate in the cells of the midgut gland, making use of nutrients which entered the cell by pino- or phagocytosis.

In *X. testudinatus* the digestive enzymes are exposed to toxin or inhibiting substances which are ingested with the food. Besides several organic substances inorganic metal-ions may act as potent inhibitors of enzyme activity. Particularly ions of heavy metals like Al, Zn, Fe, Cu, Ni, or Cd were shown to impair enzyme activities (Lopez-Lopez et al. 2003; Saborowski et al. 2004). Most digestive enzymes analysed so far are vulnerable to low pH values. Highest enzyme activities mostly appear between pH 4 and pH 8, depending on the nature of the enzyme. Typical proteolytic enzymes like trypsins show their optimum activity at slightly alkaline conditions and

are irreversibly deactivated below pH 3. In contrast a new acid proteinase was recently described in European Lobster which is most active around pH 3 (Del Toro et al. 2006). Accordingly, it appears important to investigate whether *X. testudinatus* is capable of expressing acid proteinases or other enzymes (lipases, glucanases, phosphatases) with optimum activities at acid conditions.

Elevated temperatures on one hand increase the catalytic rates of enzymes but, on the other hand, impair their stability and cause thermal degradation. The apparent thermal optimum of enzymes was often found to be above 40 or 50 °C. At these temperatures, however, most digestive enzymes already face degradation (Saborowski et al. 2004). The expression of most true thermophilic enzymes has been reported for bacteria and fungi. Only few reports are available for crustaceans from thermal vents which, however, do not functional details detail. As this hydrothermal vent crabs are exposed in their feeding grounds to high temperatures, studies on the thermal stability of their digestive enzymes appear promising.

## *1.5. Feeding and digestion in hydrothermal vent species*

The discovery of biomass rich hydrothermal vents along the Galapagos rift in the eastern pacific-ocean was one of the most exiting and important discoveries in biological oceanography of the 20th century (Lonsdale 1977; Van Dover and Lutz 2004). Soon the interests were focussed on the question of feeding and energy acquisition by organisms in this rather inhospitable habitat. The digestive system and digestive features of hydrothermal vent species provided invaluable clues in answering these questions (Van Dover et al. 1988; Boetius and Felbeck 1995). The digestive system of many sessile or sluggish deep-sea hydrothermal vent species like tubeworms clams or mussels is reduced containing tissue which carries symbionts. Some of these organisms like the polychaete *Alvinella pompejana* show high Lysozyme (muramidase) and N-acetyl-β-glucosaminidase (chitobiase) activities in midgut tissues due to a bacteriophageous feeding (Gonzáles et al. 1993). In contrast to these organism, which can be considered primary consumers, there are predatory species like the crab *Bythogrea thermydron*, feeding on vestimentiferans and mussels (Phleger et al. 2005b). This crab shows low lysozyme activities but, instead, higher activities in proteolytic and lipolytic enzymes (Boetius and Felbeck 1995).

*1.6. Lipids*

The analysis of lipids and fatty acids has proven invaluable in providing information about energy supply and feeding strategies with respect to reproduction, food scarcity, ontogeny and diapausing in marine crustaceans (Lee et al. 1974; Albers et al. 1996; Auel and Hagen 2005; Lee et al. 2006). Two major storage forms of lipids appear in crustaceans, triacylglycerides and wax esters. Triglycerides (TAG), the most common reserve lipid in animals are preferably stored to cover short term energy demands in marine copepods whereas wax esters serve as long term storage product. A specific wax-ester esterase facilitates their mobilization (Lee et al. 1972). Fatty acids which derived from triacylglycerols are useful as trophic biomarkers. The ratio of the fatty acids 18:1($n$-7)/18:1($n$-9) can provide information regarding carnivory, based on the knowledge that 18:1($n$-9) represents a fatty acid that is predominately synthesized by animals, and 18:1($n$-7) not (Dalsgaard et al. 2003). Further, many marine organisms, including copepods, diatoms, dinoflagellates and bacteria synthesize characteristic fatty acids, which are specific for those taxa. Consequently ingested fatty acids characterize fatty acid patterns of higher trophic levels and thus provide indication on a molecular basis about trophic interactions. A number of lipid profiles are available for deep sea hydrothermal vent species which rely on a bacteria based food source (Pond et al. 1998; Phleger et al. 2005a; Phleger et al. 2005b). These species show high amounts of palmitoleic [16:1($n$-7)] and vaccenic [18:1($n$-7)] acids which are generally accepted as trophic markers for sulphur oxidising bacteria in deep sea environment, where phytoplankton is less abundant (Pond et al. 2002; Dalsgaard et al. 2003; Phleger et al. 2005a). Accordingly, fatty acid analysis are suitable to complement previous stomach content analysis made by Jeng (2004) who found that *X. testudinatus* mainly feeds on pelagic copepods or other zooplankton which were killed by the toxic discharges of the vents.

Due to the spatial and temporal patchiness of food in this hydrothermal vent habitat, the ability of *X. testudinatus* to store significant amounts of lipids can be regarded as crucial in surviving prolonged periods of food depletion. It allows the animal to feed on high food abundance and utilize the endogenous lipid stores during periods of food deprivation. Effects of starvation in fish were established quite early and showed major impacts on various internal organs, mainly the liver (Steffens 1989). In crustaceans, the midgut gland (hepatopancreas) is considered to be the primary organ for lipid storage and processing (Lawrence 1976). The effects of starvation in crustaceans were intensively studied, in order to support aquaculture of

economically important species (Stuck et al. 1996; Wen et al. 2001; Oliveira et al. 2004; Wen et al. 2006; Sánchez-Paz et al. 2007). These studies have shown that the utilization of lipid, and fatty acid requirements vary in different species and along the starvation period. It was concluded, that glycogen and fatty acids display the predominant compounds in energy metabolism of crustaceans (Lawrence 1976; Sánchez-Paz et al. 2007). Furthermore, the utilization of internal proteins during prolonged starvation has been reported for decapod crustaceans (Wen et al. 2002; Kattner et al. 2003). However, the oxidation of lipids can be considered as the major energy source during prolonged starvation periods. It was concluded that fatty acid patterns and fatty acid ratios change along starvation periods indicating the utilization only preferably utilized fatty acids (Dalsgaard et al. 2003; Wen et al. 2007).

In the present thesis variations in biochemical composition, total lipid (TL) lipid class (LC) and fatty acid (FA) contents in the midgut gland of *X. testudinatus* is described throughout different stages of starvation.

## 1.7. Goals and hypotheses

The proposed work is aimed at investigating the feeding physiology and, thus, food utilization, of the hydrothermal vent crab *Xenograpsus testudinatus* at Kueishan Island,Taiwan. A major interest will be dedicated to gain a comparative picture of the feeding behaviour from both the organismic and the molecular level. These comprise the nutritive and, thus, energetic demands of the species, the characteristics and features of digestion enzymes, and the mid-gut gland fatty acid composition. The overall goal of this work is to contribute answers to the questions: how can these crabs successfully inhabit an environment otherwise hostile against live and do they show physiological or biochemical properties which can be regarded as adaptations.

The hypotheses for this investigation are outlined as follows:

*Hypothesis 1) The crabs have a low basic energy demand and show low locomotive activity.*

Observations made by Jeng et al., 2004 have revealed the very specialized feeding behaviour of this species. Only during slack tide, when the killed zooplankton falls onto the seafloor, these crabs leave their cervices, and start feeding. Usually, when there is no food available, the crabs stay in their cervices, and avoid direct exposure to the surrounding environment. This behaviour

with limited locomotive action of this species suggests relatively low basic oxygen consumption. Due to the information, that coral reefs are found on the western side of Kueishan Island, one can assume that annual temperatures are usually above 20°C in this area. As a consequence, *X. testudinatus* will show uniform metabolic changes relative to temperature ($Q_{10}$-value) within a temperature range from ~20°C to max 30°C.

*Hypothesis 2) The crabs express a set of few but highly active enzymes to efficiently digest the most important food items, i.e. proteins, lipids and polyglucans.*

The unique feeding behaviour of *X. testudinatus* can be generally characterized by inconstant food availability. During slack water, when the killed zooplankton is available, the crabs swarm out and start feeding. In order to avoid long exposure periods to the toxic environment the crabs feeding time might be as short as possible. A consequence of this behaviour might be a highly efficient digestion. As the food is composed of zooplankton several main compounds need to be efficiently digested. Proteins and lipids can be considered as the most important compounds. Additionally high chitinolytic activity can also be assumed as a central digestive feature of these crabs, due to the fact that their main food source consists of copepods (Jeng et al. 2004).

*Hypothesis 3) The digestive enzymes of the crabs show elevated tolerance against inorganic inhibitors, and maybe also against thermal degradation.*

The ability of *X. testudinatus* to survive in this inhospitable habitat needs specialized behavioural and physiological adaptations. One of these is the tolerance against sulphides, metal ions and maybe also heat. Sulphides can be considered as the most toxic compound at this hydrothermal vent site. A simple possibility in order to avoid sulphide poisoning by $H_2S$ is an alkaline internal pH that causes a shift in the equilibrium to the less toxic $HS^-$ (Goffredi et al. 1997). According to this information an alkaline pH optimum could be assumed for digestive processes. Detoxification mechanisms, including active transport of xenobiontics out of tissues requires high energetic costs. If an organism is exposed to such conditions for long times one can assume, that from the energetic point of view a general basic adaptation on the protein/enzyme level would be more economic than a steady investigation of energy on detoxification.

*Hypothesis 4) The crabs are capable of storing significant amounts of lipids to overcome prolonged starvation periods. Additionally fatty acid markers will underline the rather carnivorous feeding of* X. testudinatus.

Regarding the inconstant food supply, mainly depending on tides, winds and local currents, prolonged periods of starvation can be assumed. Facing such conditions the storage of lipids can be assumed to display an essential feature to survive in this habitat. It is hypothesized that during short-term starvation triglycerides may serve as the only energy supply and show a decrease during starvation. High amounts of the 18:1($n$-9) fatty acid may indicate carnivorous feeding. Furthermore, the tropic marker concept might provide information about the utilization of bacteria as food source in terms of high amounts of fatty acids from the MUFA ($n$-7) series.

## 2. Materials and Methods

### 2.1. Collection of crabs

Experiments with living specimens were conducted at the Institute for Biodiversity, Academia Sinica, Taiwan during summer 2007. For these investigations three hundred male *X. testudinatus* specimens with carapace widths ranging from 2 cm to 3 cm, were collected by scuba diving at the hydrothermal venting area near Kueishan island (Fig. 1). Animals were collected and transported to the Academia Sinica in aerated cooling boxes, in order to avoid overheating due to air temperatures as high as 38°C. Specimens were maintained in 250 l tanks, filled with natural seawater (Salinity: 33-35) and equipped with external Pump (Alife, AE-1060) and a bottom gravel filter system. Animals were kept under a 12 h of light and 12h of dark regime at an ambient room temperature of approximately 24 °C. Crabs were fed once daily with thin slices of Tilapia meat.

For starvation experiments 20 freshly caught specimens were sacrificed by cooling on ice. The mid gut glands were dissected, weighed, and immediately frozen in liquid nitrogen. Another 80 specimens were maintained in isolated jars to avoid aggression or cannibalism. The jars were placed in 60 l tanks which were equipped with external filters (Alife, AE-1060). Every ten days specimens from these starvation tanks were dissected. The mid gut glands were weighted in order to determine changes of the hepatopancreas index (HI) and tissue samples were stored at -80 °C for later enzyme and lipid analysis.

### 2.2. General dissection and SEM observations of the foregut ossicles

For anatomical studies of the cardiac stomach of *X. testudinatus* eight specimens were dissected using a stereo microscope (Zeiss, Stemi SV-11) and the cardiac stomach was isolated. Thereafter, sections through all three planes were made and samples were treated according to SEM protocol, including prefixation in 4% paraformaldehyde with 5% glutaraldehyde (P4G5) for 10 h. Then, the samples were transferred into a $0.1 \text{ mol·l}^{-1}$ phosphate buffer (PB) and washed three times. In order to maintain cellular structures a membrane fixation was performed with 1% $OsO_4$ in $0.1 \text{ mol·l}^{-1}$ PB for 30 min under the hood. After fixation the samples were washed in $0.1 \text{ mol·l}^{-1}$ PB again. For dehydration the samples were exposed to increasing concentrations of

ethanol (50%, 70%, 80%, 95% and 100%). The samples were dried in a critical point drier (Hitachi HCP-2 CPD), gold coated (Cressington Sputter Coater 108), and observed in a scanning electron microscope (FEI Quanta 200) within an electrical field of 25 kV.

## 2.3. Respiration measurements

For determination of oxygen consumption chambers with a volume of 5.8 l were used. Five of them were filled with filtered seawater (0.2µm) and equipped with one crab each, while another chamber served as a control. Crabs were incubated for 5 to 12 h in the dark. Reduction of oxygen concentrations below a critical level of 70 % was avoided. After incubation water samples were carefully taken and the amount of soluble oxygen was determined by the Winkler-method. In order to keep the incubation temperature constant test chambers were placed in an 80 l water bath equipped with a Dixell Prime temperature control device which was connected to the circulation incubator (Dixell, Superflite).

In order to determine oxygen consumption of bacteria derived from the crabs, chambers were carefully refilled with filtered seawater and aerated for several hours to obtain equal oxygen saturations. The chambers were again incubated for several hours and the oxygen concentrations were determined. The amount of oxygen consumed by bacteria was subtracted from the overall oxygen consumption.

## 2.4. Gut transit times

Gut transit times were determined in 20 specimens of *X. testudinatus* at 15, 20, 25, and 30°C. The animals were acclimatized to each incubation temperature for two days and were not fed during this time. Then, the animals were fed with commercial food-pellets for thirty minutes and left in jars inside the same incubation tank as used for respiration measurements. The appearance of faeces was recorded for each animal, and the average gut transit time was calculated.

## 2.5. Enzymes

Deep frozen samples were shipped in a Harsco, CX Container from the Academia Sinica, Taiwan, to the laboratories of the Marine Station on Helgoland, Germany. Samples were transferred to -80°C fridges and prepared for further analysis as described in the following.

### 2.5.1. Preparation of the crude enzyme extract from mid gut glands

Samples of frozen mid gut glands of *X. testudinatus* were weighted and extracted in 1 ml of distilled water. Homogenization of the tissue was achieved by an ultrasonic cell disruptor (Branson Sonifier, Cell disruptor B15), with 45% of maximum energy and three bursts of 3 seconds each, and a break of 7 seconds in between. The homogenates were centrifuged for 15 minutes at 15,000 g and 4°C. Thereafter, the supernatant was transferred in new reaction cups and used for further enzymatic analysis. All steps were performed on ice in order to avoid thermal degradation or enzymatic proteolysis.

Protein concentrations of the crude extracts were determined according to Bradford (1976), using a protein assay kit (Bio-Rad, Richmond, CA), with a BSA (Bovine serum albumin) standard. All measurements were performed in triplicates.

### 2.5.2. Api Zym system tests

The Api Zym system (bio Mérieux, REF 25200) was applied in order to screen *X. testudinatus* mid gut gland extracts for the following nineteen digestive enzymes: Esterase (C 4); Esterase Lipase (C8); Lipase (C 14); Leucin arylamidase; Valin arylamidase; Cysteine arylamidase; Trypsin; α-chymotrypsin; α-galactosidase; β-galactosidase; β-glucuronidase; α-glucosidase; β-glucosidase; N-acetyl-β-glucosaminidase; α-mannosidase; α-fucosidase; Alkaline phosphatase; Acid phosphatase; Naphtol-AS-BI-phosphohydrolase. The crude extracts had a concentration of 50 mg midgut gland per ml. Fifty μl of the extracts were added to each well on the test trays. The trays were incubated at 30°C for 2h and subsequently one drop of the reagents Zym A and Zym B were added to the wells. According to the intensities of the colours the enzyme activities were classified semi-quantitatively. Assays were run in dublicates for fed and

starved animals. This test is not sensitive for crustacean chymotrypsin activity due to the substrate N-glutaryl-phenylalanin-2-naphtylamid.

### 2.5.3. Lysozyme assay

Lysozyme activity of the tissue extracts was investigated according to Weisner (1984) using *Micrococcus Iuteus* lyophilisate (pH 6.3) as substrate and lysozyme from chicken eggwhite as standard. Lysozyme activity was quantified as a direct proportional decrease of absorbance at 450 nm. Measurements were performed at 30° using a (Perkin Elmer, Lambda 12)

### 2.5.4. Temperature, thermal stability, pH stability, metal ions and EDTA effects on X. testudinatus proteases

Total protease was determined using Azo-Casein Sodium salt as substrate. Measurements were performed in triplicates and two controls in order to determine background absorbance. These controls contained all reagents as described below, with the exception that crude extract was added after stopping the reaction with TCA. The assays were carried out with 20 µl of crude enzyme which were added to 200 µl of 0.1 mol·l$^{-1}$ Tris/HCl buffer (pH7). After five minutes of pre-incubation 50 µl of substrate were added and incubated for 30min at 30°C. The reaction was stopped by adding 500 µl of TCA solution (8% w/v in A. dest). The samples were centrifuged at 15,000 g for 15 min and at 4°C. The absorbance of the supernatant was read at 366nm in a photometer (Beckmann, DU 650). The absorbance of controls was subtracted from the absorbance of the assays.

The serine proteases trypsin and chymotrypsin were determined kinetically using the substrates N-Benzol-L-arginine-4-nitroanilide-hydrochloride (BAPNA; Fluka, 12915) for trypsin and N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide (SAAPNA; Sigma, S7388) for chymotrypsin. Twenty µl of crude extract were added to 960µl of 0.1 mol·l$^{-1}$ Tris/HCL buffer (pH7) and pre-incubated for three minutes. The reaction was started by addition of 20 µl of substrate. Measurements were carried out in a photometer (Perkin Elmer, Lambda 12) at a wavelength of 405nm at different temperatures.

The enzyme activities were determined at 5, 10, 15, 20, 25, 30, 40, 50, 60 and 70°C. The thermal stability of the enzymes was examined in pooled extracts from 4 individuals. Subsamples were pre incubated for 0, 0.5, 1, 2, 3, 4, 5 hours at 0, 20, 40, 50, 55, 60, 65 and 70°C. Thereafter,

samples were stored on ice and the residual protease activity was determined as described above. The effect of inorganic inhibitors on the activities of proteolytic enzymes was examined with the following metal ions and reagents: $CuCl_2$, $LiCl_2$, $CoCl_2$, NaCl, $FeCl_2$, $AlCl_2$, $MgSO_4$, $HgNO_3$ and EDTA. The concentration of all salts and reagents in the reaction mixtures was 0.01 $mol \cdot l^{-1}$.

The pH-profiles of proteases were investigated using universal buffer as described by Ellis(1961). The pH stability was studied whilst crude extracts were incubated at pH 2, 4, 6, 8, 10, 12 for 30 min and at 30°C. Subsequently, the samples were examined for protease activity at pH 7. The pH-optimum was determined by measuring enzyme activity at each of the aforehead mentioned pH.

### 2.5.5. *Measurement of lipolytic activity*

The lipolytic activity of *X. testudinatus* mid gut gland extracts was determined using 4-Methylumbelliferyl butyrate (MUF; Fluka, 19362), an artificial fluorescent substrate. Ten μl of extract were pre-incubated in 490 μl of Tris/HCl-buffer (0.1 $mol \cdot l^{-1}$, pH 7) for one minute. Thereafter, 10 μl of substrate were added. The increase of fluorescence was measured continuously for three minutes at 360 nm excitation and 450 nm emission (Kontron Instruments; SFM 25). All measurements were performed in doublicates. The change of fluorescence is direct proportional to the degradation of substrate by lipase/esterase and activities were expressed as units/$g_{FM}$ per minute.

### 2.5.6. *SDS-Page assays for proteins, protease activity and lipase activity*

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). The crude extracts with adjusted protein concentrations (see legend, Figure 13) were diluted (1:2) with sample buffer containing SDS. For protease and lipase/esterase activity gels 10 μl of sample were loaded into the gel pocket and gels were run at 300 V and 15 mA per gel. After electrophoresis protease gels were soaked in universal buffer (pH 4, 8 or 10) for 30 min.

Gel designated for protease activity determination were transferred to a 3% solution of casein (Sigma, C5890) or hemoglobin (Sigma, H2625), adjusted to pH 10 or pH 4. First, the gels were incubated for 30 min on ice and then for another 90 min at room temperature. Next, gels were

washed with universal buffer and Coomassie-stained (0.05% Coomassie brilliant blue, 40% Methanol and 7% acetic acid) for several hours. Finally, the gels were washed in a destaining solution (10% acetic acid and 40% methanol). Bands showing proteolytic activity appeared pale on the gel while the background was dark blue due to undigested casein or hemoglobin.

Gel designated for lipase activity staining were first washed in 2.5% Triton solution then in 50 mmol·l$^{-1}$ phosphate buffer (pH 7) and finally incubated for 15-20 min in a solution of 100 µmol·l$^{-1}$ MUF-butyrate dissolved in phosphate buffer (pH 7). Fluorescent bands of hydrolysed substrate appeared under UV illumination indicating lipase/esterase activity. Photographs were taken under a BIO-RAD (Universal hood II) gel-chamber connected to a gel documentation system (ChemiDoc XRS) and analysed by the software Quantity One version 4.4.1 (BioRad).

The molecular weight marker used was a low range markers ranging from 6.5 to 66 kDa (Sigma, M3913).

### 2.5.7. Enzyme purification

Proteins in the mid gut gland extracts were separated by anion exchange chromatography using a FPLC-system (Pharmacia, Model no. 20-0002-02). Samples were desalted and rebuffered by loading 0.5 ml of extract onto a NAPk-10 Sephadex G25-column (AP Biotech) and eluted with 1 ml of 0.01 mol·l$^{-1}$ imidazole buffer, pH 6.8 (buffer A). This was performed twice in order to receive 2 ml of desalted sample. Afterwards, 2 ml of processed extracts were loaded onto a UNO Q1-R anion exchange column (BioRad, Hercules, USA). The bound proteins were eluted with a linearly increasing gradient of NaCl, using 0.01 mol·l$^{-1}$ imidazole buffer (pH 6.8) with 1 mol·l$^{-1}$ NaCl (buffer B). Absorbance (280nm) and conductivity of eluted proteins were detected simultaneously recorded. The flow rate was 1 ml/min and fractions of 0.5 ml each were collected. The fractions were assayed for Chymotrypsin and lipase using same substrates as described above. Fifty microliters of each fraction were transferred onto a 96-well microplate and 250 µl Substrate containing buffer was added (final concentrations of substrates were: SAAPNA, 2.2 mg·ml$^{-1}$; MUF-butyrate, 0.03 mg·ml$^{-1}$). After 5–10 min of incubation at room temperature (20-25°C), fractions showing Chymotrypsin activities were visually detected. Lipase activity was determined fluorometrically, immediately after addition of substrate, under UV light using a BIO-RAD (Universal hood II) chamber. Fractions with maximum Chymotrypsin and lipase activities, were pooled for electrophoretical protein and zymogram analysis.

## 2.6. Lipids

Deep frozen samples were transported from the Academia Sinica, Taiwan, to the University of Bremen, Germany, in cooling containers (Harsco, CX Containers) and stored there at -80°C.

### 2.6.1. Lipid extraction and gravimetrical determination of lipid content

Frozen mid gut glands were lyophilized for 48 h and the dry weight was recorded. The lyophilized samples were homogenized and extracted on ice in 4+4 ml dichlormethan/methanol (2:1 v/v, DCM) using a potter (Sartorius; Potter S), and afterwards sonicated with an ultrasonic cell disruptor (Bandelin, Sonoplus) for 30 seconds with one pulse per second. All extraction steps were performed on ice. Homogenates were transferred into centrifuge tubes and centrifuged at 2°C for 10 min at 2500 rpm (Sigma, Laboratory centrifuge, 6K15). Supernatants were transferred to new tubes and 2 ml of ice-cold 0.88% Potassiumchloride solution were added and thoroughly mixed for 30 seconds. The samples were then again centrifuged for 10 min. Afterwards the upper aqueous phase was discarded and the lower lipid-containing phase was transferred into previously weighed vials. In order to avoid oxidation of lipids the samples were evaporated under a stream of nitrogen atmosphere until all solvent evaporated (ca. 1 hour). Prior to weighing on a microbalance (Satorius precision balance R200D, precision 10μg) all samples were dried for 30 min in an exciccator. The amount of lipid was presented as % of dry weight.

For storage 1 ml DCM was added and samples were kept at -80°C.

### 2.6.2. Lipid class analysis

Lipid extracts adjusted to a final concentration of 14 mg·ml$^{-1}$ in DCM were used to determine lipid class composition by thin layer chromatography with flame ionization detection (TLC- FID). Before use, chromarods were soaked in 2.5% $NH_4OH$ for 5 min and afterwards washed in deionized water for three times. Chromarods were dried for 20 min at 100°C and flamed in order to remove old samples. 0.5 μl were applied on chromarods in doublicates. Chromatography was performed in a running chamber, containing hexane, diethylether and formic acid (85:15:0.04) for 25 min. Finally chromarods were dried at 100°C for another 20 min and analyzed using Iatroscan (Iatron laboratories, Inc., MK-5)

*2.6.3.    Fatty acid analysis*

Subsamples of lipid extracts (100 µg) were used for transesterification. After evaporation of the solvent under a continuous flow of nitrogen 1 ml of methanolic sulphuric acid (3%) and 250 µl of hexane were added to the sample and the mixture was incubated free of oxygen for 4 h at 80°C. Thereafter, 1 ml hexane and 2 ml of demineralised water were added to the sample. After shaking for 30 s the mixtures were centrifuged at 2500 rpm for 10 min at 2°C. The lipophilic phase was transferred into a new glass vial and evaporated with nitrogen. This step was repeated twice with the remaining water/hexane mixture by addition of 1 ml hexan. The dried sample was then dissolved in hexane and stored at -20 to -80°C. Fifty µl of sample were adjusted to a concentration of 0.02 mg·ml$^{-1}$ hexan and were then analysed by gas-liquid chromatography. The device (HP 6890, see Table 1) was equipped with a cold-injection-system and a DB-FFAP-column (30 m long, inner diameter of 0.25 mm, 0.25 µm coating). The carrier gas was Helium and the temperature was 80 to 240°C. FAMEs were quantified by a flame ionization detector and identified by comparison with retention times of a known standard (marinol).

*2.6.4. Statistical analysis*

Statistical analysis was performed by the computer program SigmaStat 3.1 (SPSS Inc., Chicago, IL, USA). One way analysis of variance (ANOVA), including all pairwise multible comparison procedure (Holm-Sidak method), was applied to evaluate differences of proteolytic activities under varying experimental conditions, and was additionally performed to represent activity and lipid differences along the starvation period. Students t-test was applied for comparison of fatty acids of fed and starved animals. All activities and lipid/fatty acid contents are presented as mean values including standard deviations.

Table 1  Gaschromatograph data and operating parameter

| | |
|---|---|
| gaschromatograph | HP 6890A |
| gas | helium, hydrogen, nitrogen (purity 5.0) |
| | synthetic air, carbondioxide (liquid) |
| | |
| sample injection | autosampler (MultiPurposeSampler) |
| injector | Coldinjector 3 (KAS3) |
| temperature | 25°C |
| injection volume | 10µl |
| end temperature | 350°C |

**Column**

| | |
|---|---|
| length and thickness | 30m*0.25mm |
| liquid phase | DB-FFAP |
| coating thickness | 0.25µm |
| column flow | 0.8 ml/min |
| carrier gas | helium (purity (5.0) |

**Oven**

| | |
|---|---|
| start temperature | 85°C for 5 min |
| 1. temperature gradient | 30°C/min to 165°C |
| 2. temperature gradient | 4°C/min to 240°C, hold for 1 5 min |

**Detector**

| | |
|---|---|
| | FID |
| operation temperature | 280°C |
| hydrogen flow | 30 ml/min |
| nitrogen flow | 19.2 ml/min |
| synthetic air flow | 300 ml/min |

## 3. Results

*3.1. Field observations of X. testudinatus in the natural environment*

On June 3rd, 2007 we got the unique opportunity to collect *X. testudinatus* specimens for this study and, simultaneously, to do field observations of this species in its natural habitat. The locations where the crabs were sampled were 8-15m deep. The habitat was poor in flora and fauna (Fig. 2) and was filled by the smell of sulphurous plumes discharged by the fumaroles and solfataras. Gas bubbles descending from the sea floor caused burns on skin because of their high amount of acids making the dive uncomfortable. The benthic environment was inhabited by few anthrozoan anemones, snails (mainly *Nassarius sp.*), and some coralline red algae. A closer look, however, revealed hundreds of crabs densely packed inside of cavities, even sitting on one another. Only when we tried to capture them, the crabs started to escape. These crabs were covered by dense filamentous layers of bacteria mainly seen on the base of their legs and their mouthparts. These bacterial mats disappeared after several days when the animals were maintained in the aquaria at the Academia Sinica. In their natural habitat and also in captivity the crabs show the behaviour of grooming their legs and carapace and ingesting the removed particles. During scuba diving we observed how a group of these crabs fed on a dead flying fish which probably was killed by the toxic plumes.



Fig. 2  Crabs, *Xenograpsus testudinatus*, hiding densely packed in their sulphurous crevices avoiding the toxic waters discharged by the vents (left). During slack water crabs start to swarm out for their search for food (right). The entire environment was poor of life and a strong smell of sulphur filled the water.

*3.2. Anatomy and structure of the foregut ossicles*

*Xenograpsus testudinatus* is a grapsid crab and shows the typical morphological features of this family. The carapace is dorso-ventrally flattened and the walking legs are posteriorly angled. Males can reach a maximum carapace width of 3 cm and they bear a pair of large claws. In contrast, the claws of females are much smaller and more ventrally directed. As in all brachyuran families the bythograeid foregut, as viewed from dorsally, is a triangular, trapezoidal, or heart-shaped membranous sac supported and shaped by cuticular components of various thickness and complexity (Martin et al. 1998). The general descriptions of grapsid foreguts apply for *X. testudinatus*, too. The nomenclature of components labelled and the terminology of the description of the foregut widely follows Martin et al. (1998).
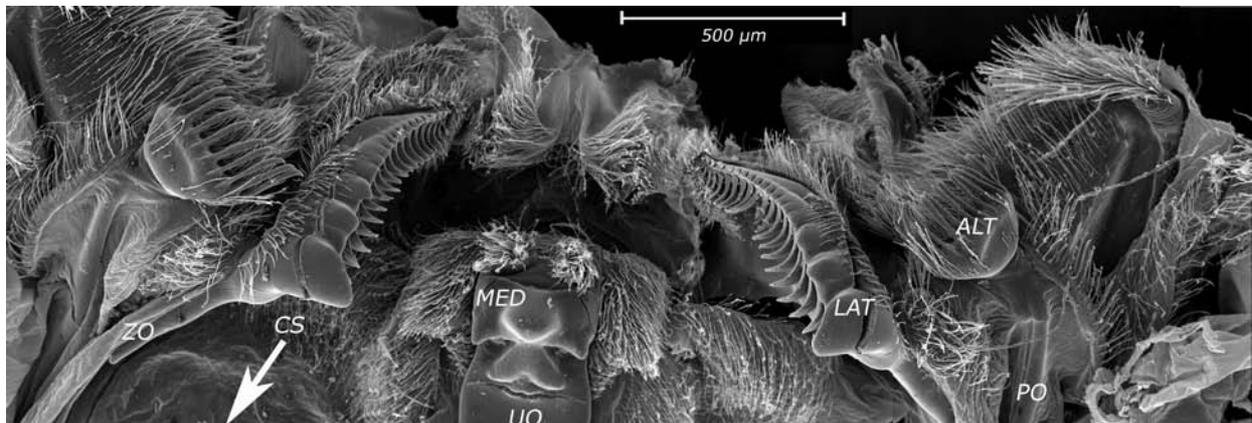


Fig. 3 SEM image of the internal anatomy of the stomach of *X. testudinatus*. Dorsal view on the open stomach revealing the median tooth (MED), the lateral tooth (LAT), accessory lateral tooth (ALT), as well as the urocardiac ossicle (UO), zygocardiac ossicle (ZO), and the prepectineal ossicle (PO). The position of the cardiac stomach (CS) is indicated by the arrow.

The median tooth of *X. testudinatus* can be separated in two parts: a larger rectangular part with a large central dorsal projection and a smaller part that is V-shaped. The median tooth is surrounded by setae of different length with two groups of striking projecting setae at the posterior edge of the tooth (Fig. 3 and Fig. 5 (a)). The urocardiac ossicle is smooth, and remains of a tongue carrying the median tooth on its posterior end. The lateral teeth are well developed and carry one large tooth at their anterior end. The posterior part consists of ~ 18 blade shaped teeth, which become smaller in posterior direction. Along the ventral margin of the lateral tooth and the posterior end several setae are present. These setae, like most of the other setae found in the internal stomach of *X. testudinatus,* are denticle bearing (Fig. 5 (e, f)). Accessory teeth are paired, attached to prepectineal ossicle, and located dorsally in the anterior part of the lateral teeth

(Fig. 3, Detail: Fig. 5 (b)). These accessory ossicles bear eleven medially directed spines which carry denticles at their end. Only few setae are present on the anterior surface of the accessory teeth. However, on the posterior side these scaled setae form dense filters. The entire foregut is densely equipped with mainly two types of setae. The first type consists of a central slightly fattened body carrying two rows of lateral directed spines. These spines only occur at the tip of the setae. This type is found along the dorsal edge of the lateral teeth (Fig. 5 (e)). The second type of setae is cylindrical carrying small denticles. These denticles cover one side of the round setae (Fig. 5 (f)). The second type is widely distributed throughout the whole foregut.

### 3.3. Respiration measurements and transit times

The routine oxygen consumption (ROC) of *X. testudinatus* at the ambient temperature of 25°C was 3.5±1.0 [$\mu mol \cdot g_{FW}^{-1} \cdot h^{-1}$]. Respiration increased from 1.1 ± 0.2 [$\mu mol \cdot g_{FW}^{-1} \cdot h^{-1}$] at 15°C to 4.9 ± 1.5 [$\mu mol \cdot g_{FW}^{-1} \cdot h^{-1}$] at 30°C. The ROC increased exponentially with temperature. The $Q_{10}$- value calculated over the whole temperature range of 15, 20, 25 and 30°C was 3.13. $Q_{10}$-values for the temperature range of 20 to 25 and 25 to 30 were approximately 2 (Fig. 6).

Between 20 and 15°C the $Q_{10}$ increased two to three fold ($Q_{10}$ = 5.43).
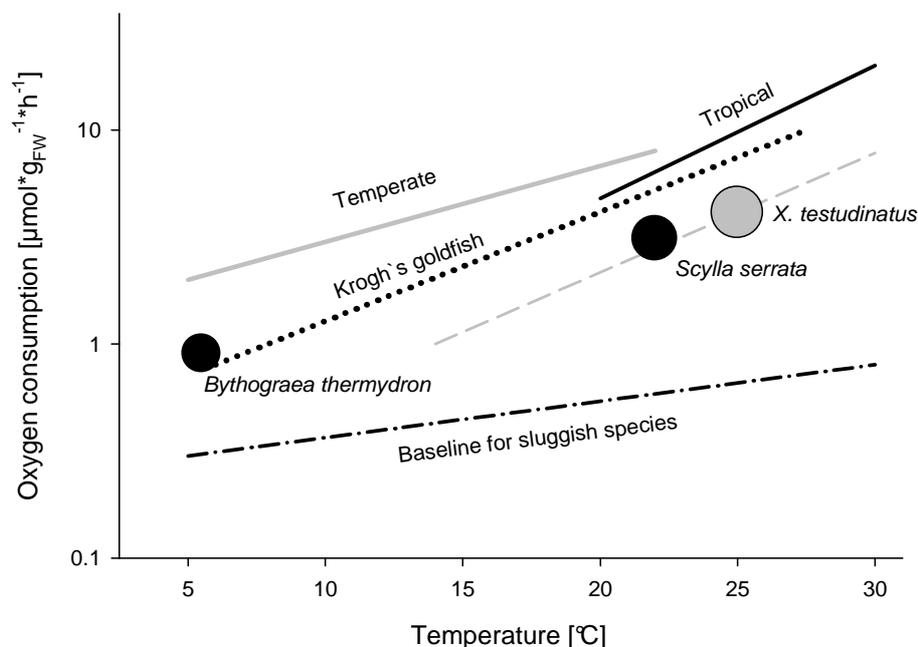


Fig. 4 The Krogh`s curve modified after Kunzmann et al. (2007), including the respiration curves of *X. testudinatus* (grey dashed line) and the oxygen consumption of the deep sea hydrothermal vent crab *Bythograea thermydron*.
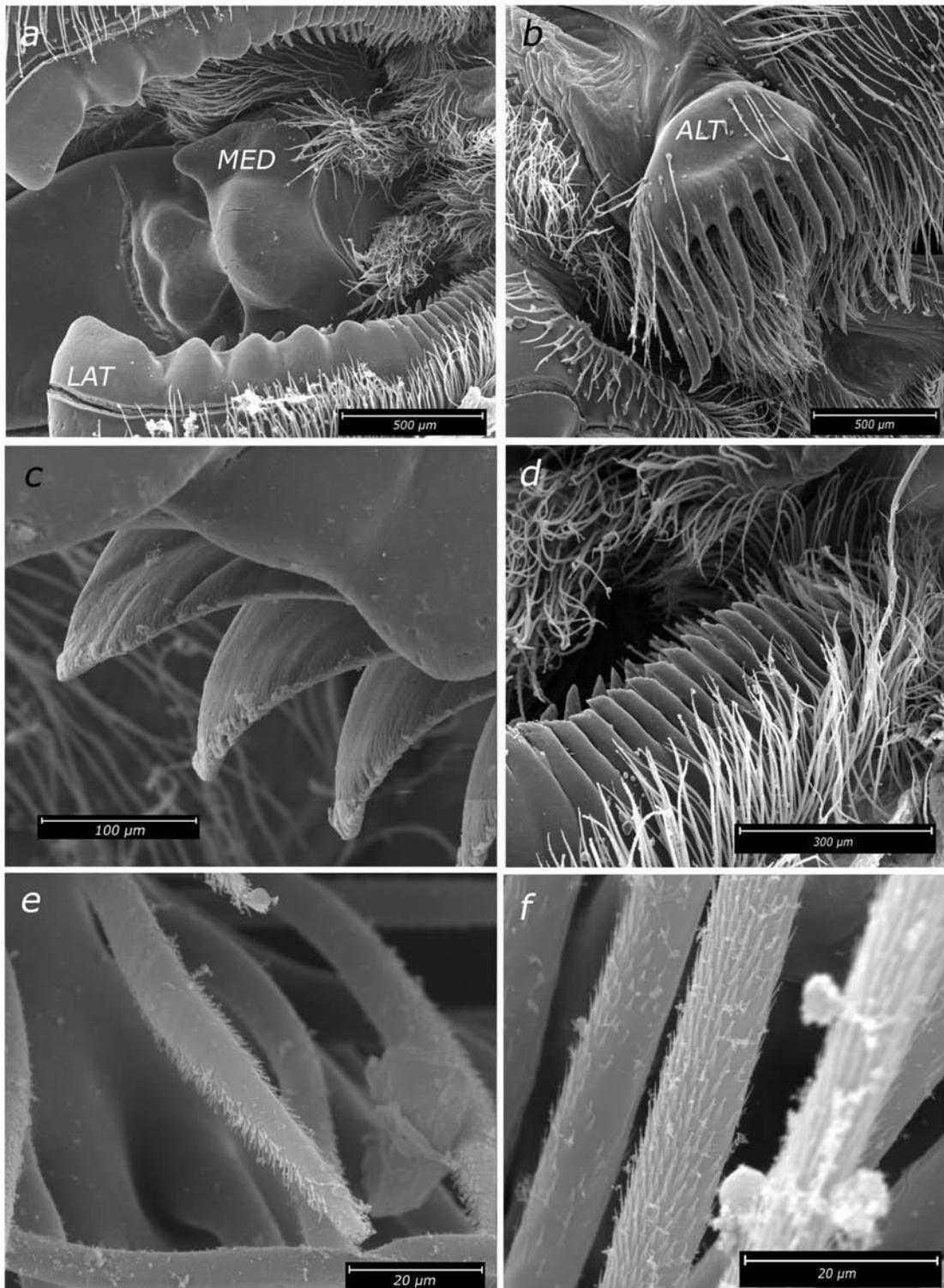
Fig. 5 SEM photograph of the foregut ossicles and the setae covering the internal surface of the foregut. The lateral teeth and the median tooth (a) note the setae covering the dorsal surface of the lateral tooth. Close up of the accessory teeth from anterior view, showing the 11 medially directed spines (b). Detailed image of the blade shaped lateral teeth from dorsal view (d) and from lateral view (c). Images (e) and (f) revealing the fine structure of the setae, showing two types of setae, differing in their denticle distribution.

Measurements of oxygen consumption allow for the calculation of released $CO_2$ and, consequently, reveal information about energetic demands. Assuming a respiratory quotient of 0.85 and an ambient temperature of 25°C, *X. testudinatus* specimens release 2.74 µmol $CO_2 \cdot g_{FW}^{-1} \cdot h^{-1}$. This equals to 7.89 mg $C \cdot 10g^{-1} \cdot d^{-1}$. According to Salonen et al. (1976) the amount of energy stored in 1 g organic carbon (Zooplankton) corresponds to 41.4 ± 0.9 kJ (9.9 kcal). Accordingly, the daily energy demand of an specimen with 10 g body mass amounts to 0.326 kJ, or 326 J (0.078 kcal).
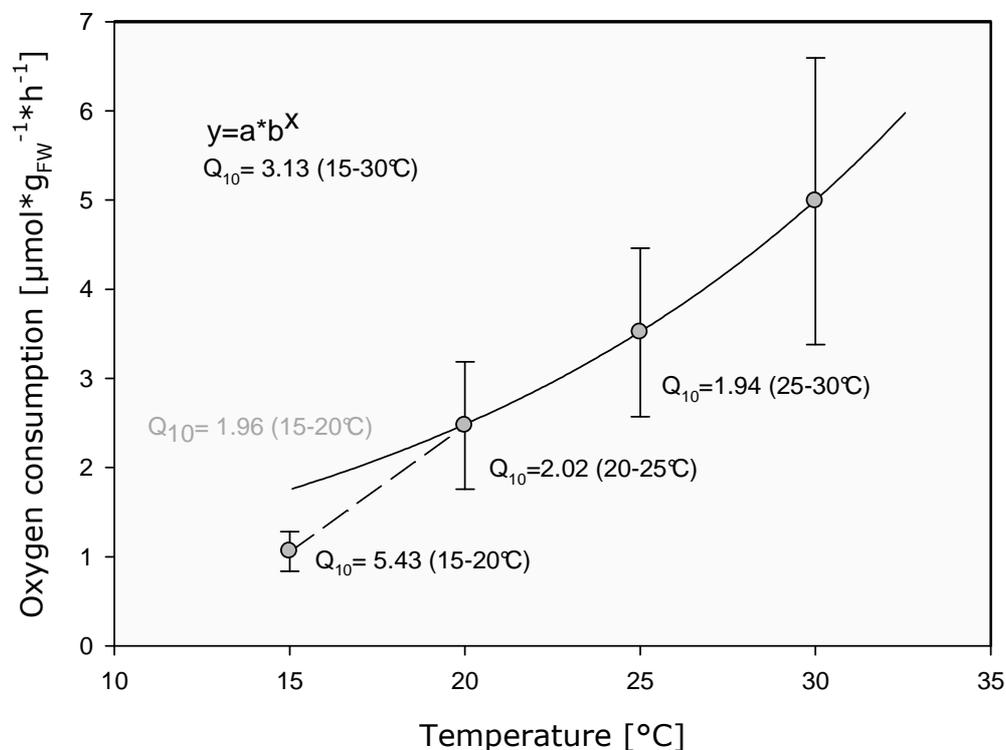


Fig. 6 Temperature dependent oxygen consumption of *X. testudinatus* . Between 20°C and 30°C an exponential regression was applied in accordance to the van t`Hoff`s law (solid line). Oxygen consumption rates at 20 and 15°C are connected by a dashed line indicating a deviation from the exponential course. $Q_{10}$-values are given for each temperature level tested and additionally for a theoretical value between 15 and 20°C (grey). Error bars indicate standard deviations, (n=9-14).

The digesta transit time of *X. testudinatus* ranged from 3.13 to 8.17 hours depending on incubation temperature. The temperature dependency can be characterized by decreasing transit times with increasing temperatures (Fig. 7). At 15°C animals showed just little, or almost inhibited food ingestion.
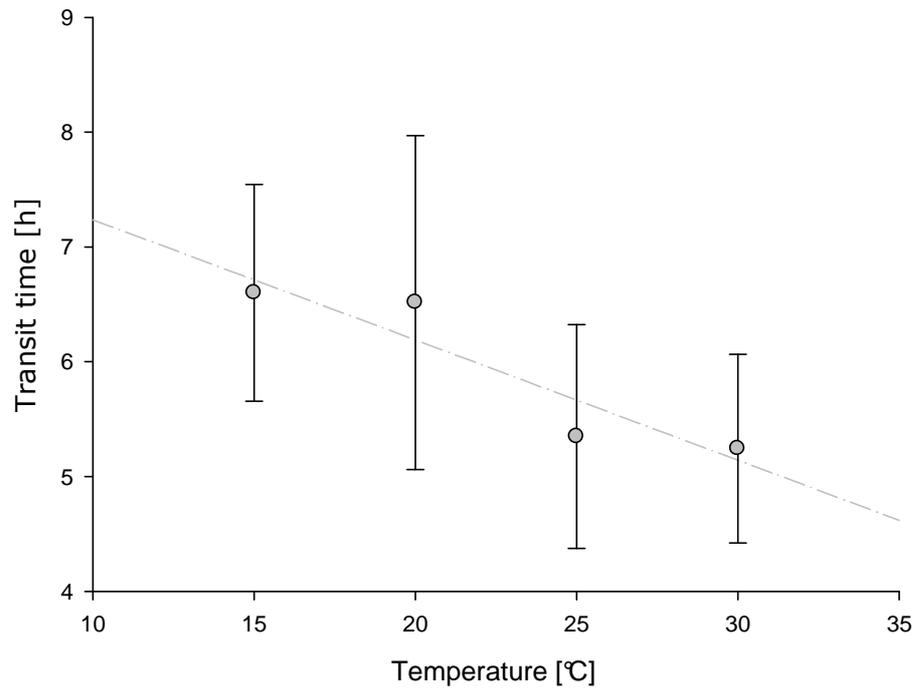
Fig. 7 This plot shows the temperature dependent gut transit times of *X. testudinatus*, revealing an enhanced gut passage with increasing temperature. Bars indicate standard deviations (n=10).

## 3.4. Enzymes

### 3.4.1. Api Zym system tests

The semi-quantitative enzyme screening of mid gut gland extracts of *X. testudinatus* revealed a wide range of enzyme activities (Table 2). Almost all enzymes tested showed positive reactions except α-chymotrypsin and Lipase (C-14). Highest activities, scaled 5, showed Leucin arylamidase, Trypsin, α-glucosidase and alkaline phosphatase. Phosphate hydrolase, including alkaline phosphatase, acid phosphatase and Naphtol-AS-BI-phosphohydrolase, showed high activities as well. All glucosidases tested showed positive intermediate reactions.

Table 2 Enzyme activities determined with the semi-quantitative Api Zym system for freshly caught, and for starved specimens. Activities were determined visually by strengths of the coloration and ranked in activity levels ranging from 0 for no reaction to the maximum intensity of 5. Chymotrypsin was negatively tested due to a non suitable substrate (N-glutaryl-phenylalanine-2-naphtylamide) for brachyuran chymotrypsin.

| Enzyme | colour intensity | Enzyme | colour intensity |
|---|---|---|---|
| Ester hydrolases | | Phosphoric hydrolases | |
| Esterase (C 4) | 2 | Alkaline phosphatase | 5 |
| Esterase Lipase (C8) | 3 | Acid phosphatase | 4 |
| Lipase (C 14) | 0 | Naphtol-AS-BI-phosphohydrolase | 4 |
| Glucosidases | | | |
| α-galactosidase | 1 | | |
| β-galactosidase | 4 | Peptide hydrolases | |
| β-glucuronidase | 4 | Leucin arylamidase | 5 |
| α-glucosidase | 4.5 | Valin arylamidase | 3 |
| β-glucosidase | 3 | Cysteine arylamidase | 2 |
| N-acetyl-β-Glucosaminidase | 5 | Trypsin | 5 |
| | | α-chymotrypsin | 0 |
| α-mannosidase | 3 | | |
| α-fucosidase | 4 | | |

### 3.4.2. Temperature, thermal stability, pH, metal ions and EDTA effects on X. testudinatus proteases

The total activities measured at 30°C were $9.2\pm3.3$ $U\cdot min^{-1}\cdot g^{-1}_{FW}$ for Trypsin and $68.3\pm11.1$ $U\cdot min^{-1}\cdot g^{-1}_{FW}$ for Chymotrypsin. The effects of temperature on mid gut gland proteases were investigated in two different respects. On the one hand enzyme activities were determined at various temperatures from 5 to 80 °C to determine the temperature optimum of the enzymes. On the other hand the enzymes were incubated at various temperatures and for different duration in order to examine their thermal stabilities. In general the temperature/activity curve showed an exponential increase of activity from 5 to 50°C for both trypsin and chymotrypsin (Fig. 8 (a)). Chymotrypsin showed maximum activity at 50°C and Trypsin at 60°C (Fig. 8 (b), (c)). At 70°C, however, both enzymes lost almost their entire activities.

The residual activities of trypsine, chymotrypsine and total proteases after thermal exposure are presented in Fig. 10. The thermal stability tests revealed no loss in activity for trypsin, chymotrypsin and total protease up to 50°C and up to an exposure time of 5 h. All enzymes tended to increase activities at 40 °C exposure temperatures. At 5 hours of incubation at 55°C

chymotrypsin started to denature loosing about 40% of initial activity. In contrast, trypsin showed elevated activities even at 60°C slowly declining to initial levels after 5h of incubation. Significant thermal degradation of trypsin started at 65°C. After 5 hours of exposition the activity dropped to 30% of initial values.
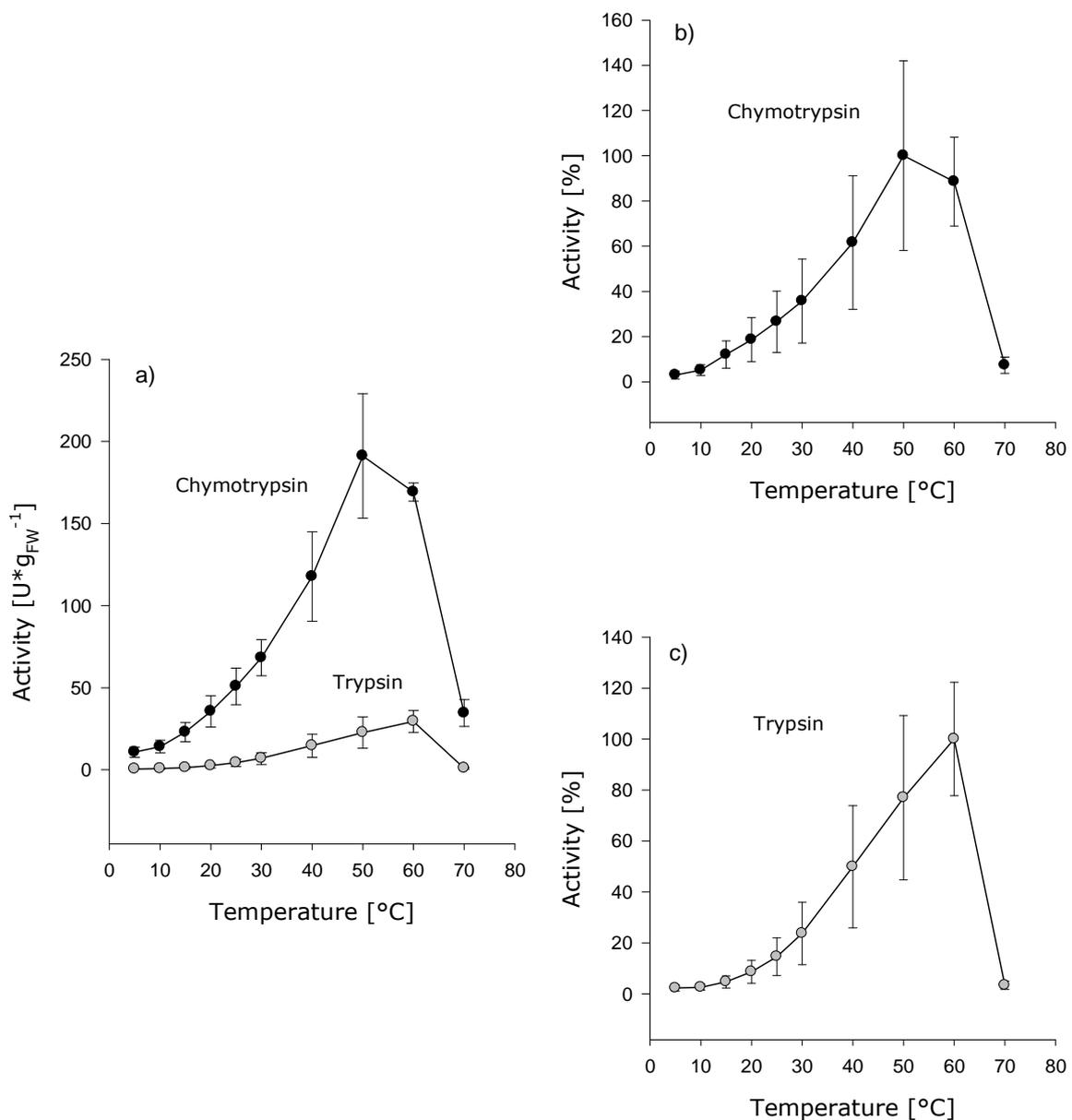


Fig. 8 Temperature dependent activity curves for the proteases Trypsin and Chymotrypsin. Plot (a) compares the activity curves of both enzymes, given in activity units per g fresh weight (U·$g_{FM}^{-1}$). Plots (b + c) represent temperature dependent activities in % of the highest activity.

The effects of different pH on protease activities were investigated using universal buffer adjusted to pH ranging from 2 to 12. The pH optimum and the pH stability were examined (Fig. 9 (a), (b)). The pH stability of Trypsin and Chymotrypsin showed similar courses. After 30 min of incubation the residual activities at pH 2 were less than 10% but amounted to 65% at pH 4. Maximum stability was present between pH 6 and pH 10. At pH 12 the residual activity of trypsin almost vanished and chymotrypsin activity decreased to approximately 50% of maximum. Determination of the pH optimum revealed maximum activity of chymotrypsin at pH 8 using SAAPNA as substrate and maximum activity of Trypsin at pH 10, using the substrates BAPA. Below pH 6 almost no activity was detectable. From pH 4 to pH 8 an almost linear increase was observed peaking at pH 10 for Trypsin and at pH 8 for Chymotrypsin. The activity measurements at pH 12 were not reliable due to the degradation of both substrates at this pH. The pH stability of the substrates was investigated by measurements without the addition of the crude extract, and there was no change in absorbance observed at pH 10 indicating a stability of these substrates at this pH.
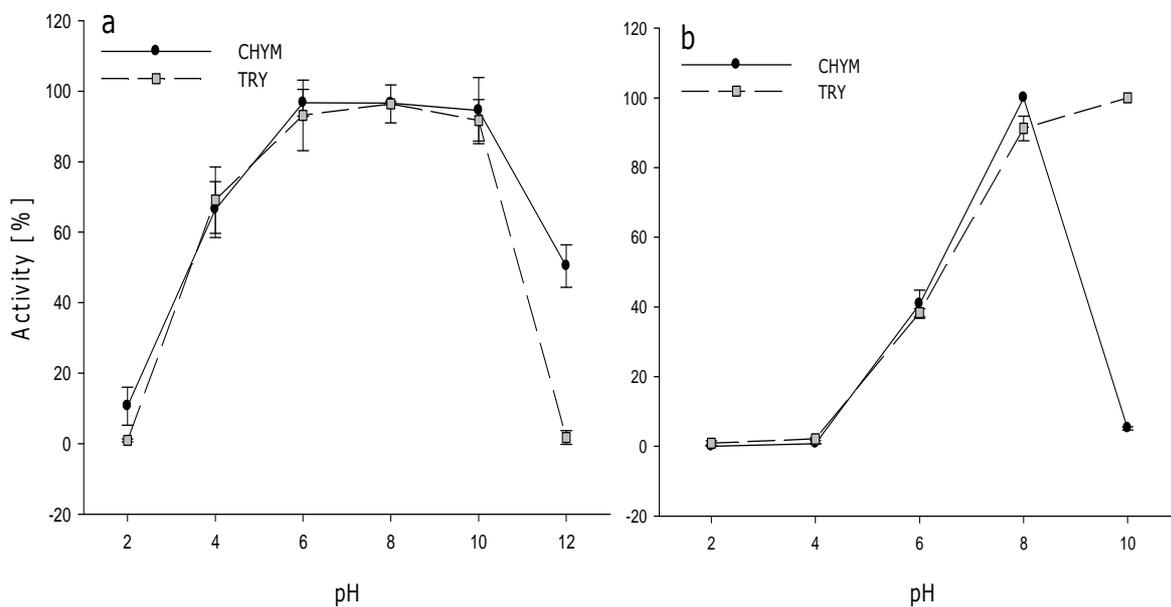


Fig. 9 Effects of pH on protease stability and the pH optimum were investigated using universal buffer. The residual activity of pH stability of trypsin (TRY) and chymotrypsin (CHYM) (a) and the pH optimum of both are presented in relation of the maximum activity. The substrates BAPA and SAAPNA showed degradation effects at pH 12 and thus, not presented in the pH optimum plot.
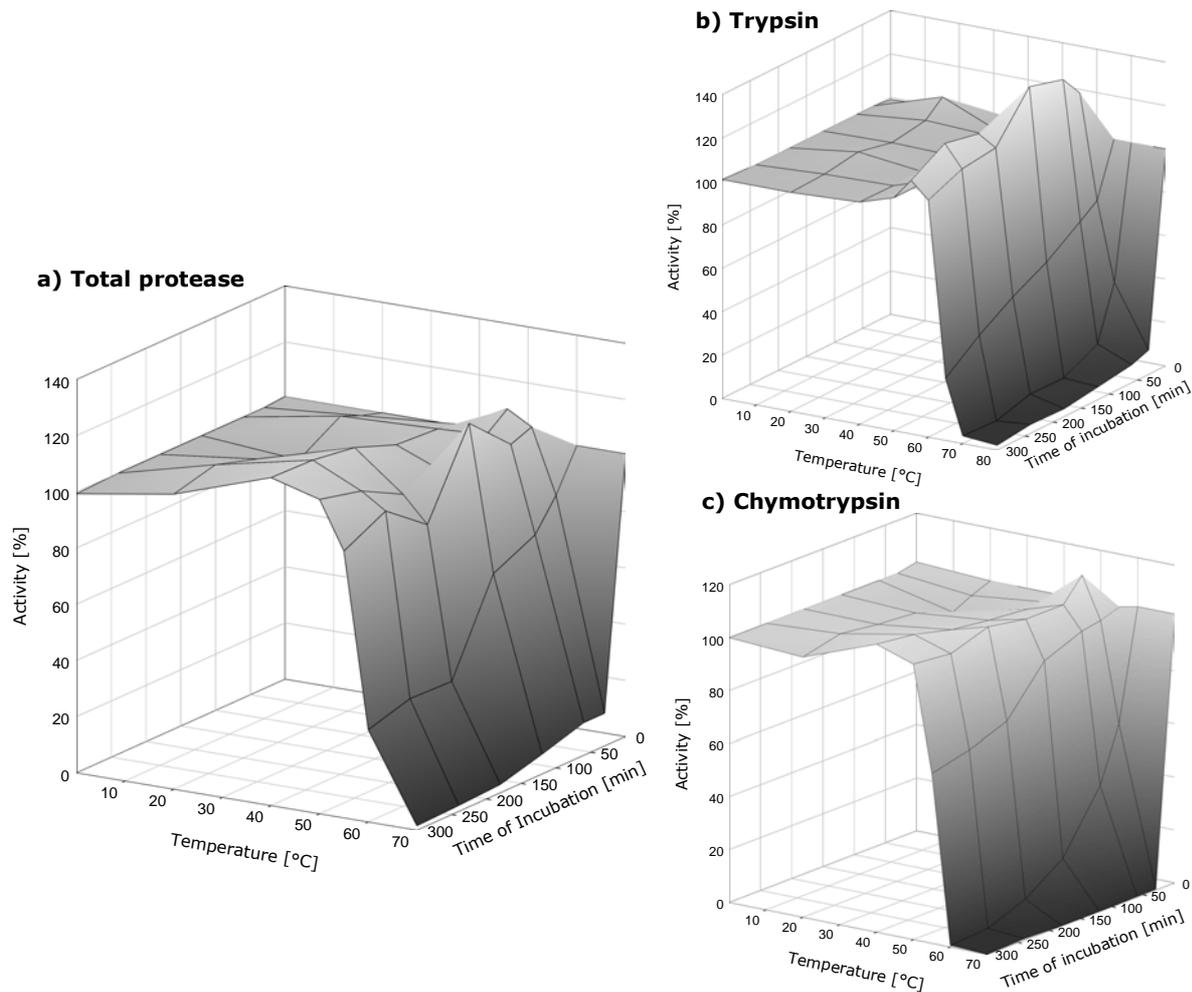
Fig. 10 Thermal stability of total protease, trypsin and chymotrypsin. Samples from pooled extracts (n=4) were incubated at temperatures ranging from 0 to 80°C and exposed to the respecting temperature for 0 to 5h. Subsequently, activity assays were run at 30 °C. The residual activity is given as percentage of the activities measured from samples kept on ice.

Studies on the effects of metal ions and EDTA on proteolytic activity revealed similar effects for trypsin and chymotrypsin (Fig. 11). The assays with $LiCl_2$, $NaCl$, $NaSO_4$, $MgSO_4$ and $HgNO_3$ showed no decrease in activities whereas $CuCl_2$, $CoCl_2$, $AlCl_2$, $FeCl_2$ and EDTA inhibited proteolytic activities. Inhibition by copper ions was strongest leaving residual activities of 20 to 30%. Iron reduced activities by 40 to 60%. Cobalt and EDTA showed low inhibition with remaining activities of 80%. $AlCl_2$ had a different effect on trypsin than on chymotrypsin. Trypsin was reduced to 50% of remaining activity whereas chymotrypsin remained 80% of its activity. Additionally effects of sulphurous compounds as sulphide, sulphate and sulfite were tested, but these compounds caused no significant inhibition.
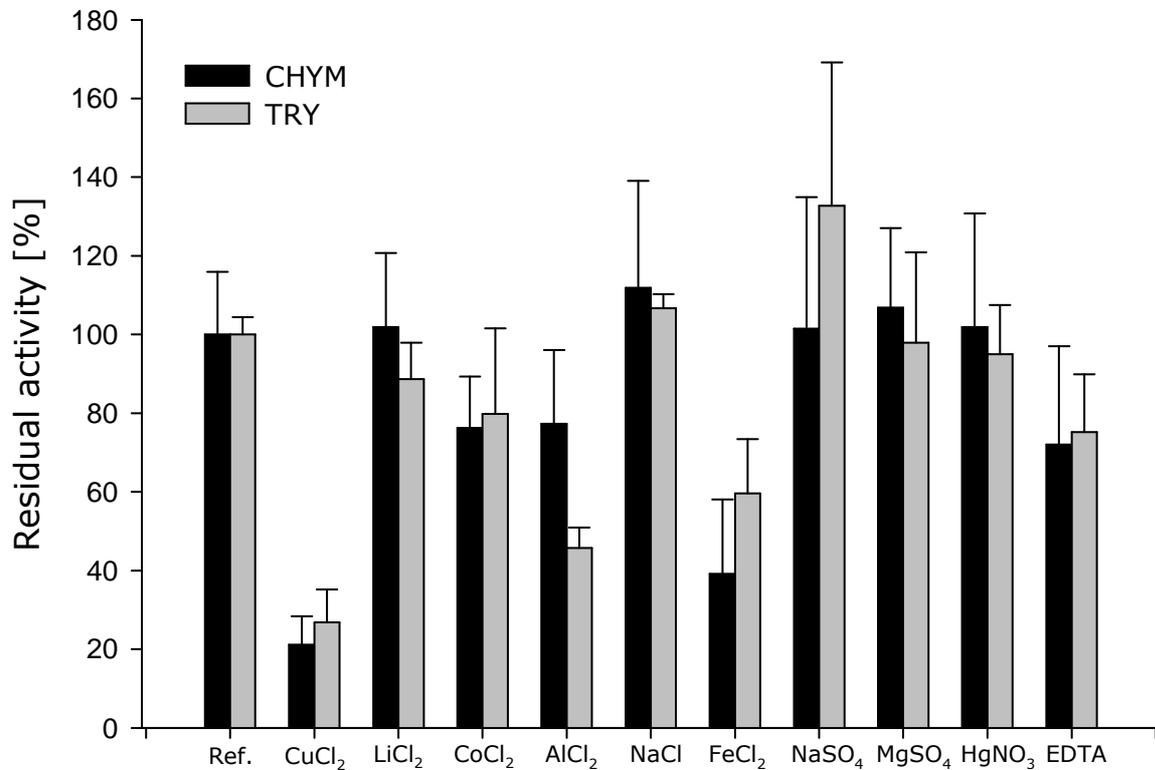
Fig. 11 The effects of metal ions and EDTA on trypsin (TRY) and chymotrypsin (CHYM) activities. Residual activities are presented in % of uninhibited activities. (Mean ± SD, n = 3)

Table 3  Effects of inorganic inhibitors on trypsin and chymotrypsin

| Trypsin | | | Chymotrypsin | | |
|---|---|---|---|---|---|
| | Activity [dE/min*gFW] | Difference | | Activity [dE/min*gFW] | Difference |
| Ref. | 2.309 ± 0.102 | a | Ref. | 18.643 ± 2.972 | a |
| $CuCl_2$ | 0.619 ± 0.193 | c | $CuCl_2$ | 3.939 ± 1.351 | c |
| $LiCl_2$ | 2.046 ± 0.214 | b | $LiCl_2$ | 19.000 ± 3.511 | a |
| $CoCl_2$ | 1.843 ± 0.502 | ab | $CoCl_2$ | 14.215 ± 2.433 | ab |
| $AlCl_2$ | 1.056 ± 0.120 | ab | $AlCl_2$ | 14.404 ± 3.506 | ab |
| NaCl | 2.464 ± 0.081 | a | NaCl | 20.856 ± 5.068 | a |
| $FeCl_2$ | 1.376 ± 0.318 | b | $FeCl_2$ | 7.301 ± 3.531 | bc |
| $NaSO_4$ | 3.065 ± 0.842 | a | $NaSO_4$ | 18.917 ± 6.231 | a |
| $MgSO_4$ | 2.261 ± 0.530 | a | $MgSO_4$ | 19.908 ± 3.781 | a |
| $HgNO_3^-$ | 2.194 ± 0.288 | a | $HgNO_3^-$ | 18.997 ± 5.384 | a |
| EDTA | 1.736 ± 0.340 | a | EDTA | 13.417 ± 4.673 | ab |

Residual Trypsin and Chymotrypsin activities including standard deviations during inhibition experiments. Same letters denote non- significant difference (α=0.05) between each treatment.

*3.4.3. SDS-PAGE of proteins, protease activity and lipase activity*

SDS-PAGE was performed with mid gut gland extracts of *X. testudinatus* to separate proteins as well as proteinases and lipases. Proteinase zymograms at pH 10 revealed seven activity bands of apparently 45 to 14.2 kD (Fig. 13 (b)). Strongest proteolytic activities showed three bands in the lower molecular weight range. In general, a loss of proteolytic activity was evident along with advancing starvation. A similar decrease of activity appeared in lipase activity gels which showed four major activity bands. Highest lipolytic activity was expressed by a band of apparently 24 kDa. The strongest band on the protein-activity gels, which is located slightly below the 24 kDa marker band showed proteolytic activity at pH 4 and at pH 10. Ion exchange chromatography revealed that the proteolytic and lipolytic enzymes showed similar elution characteristics (Fig. 12).
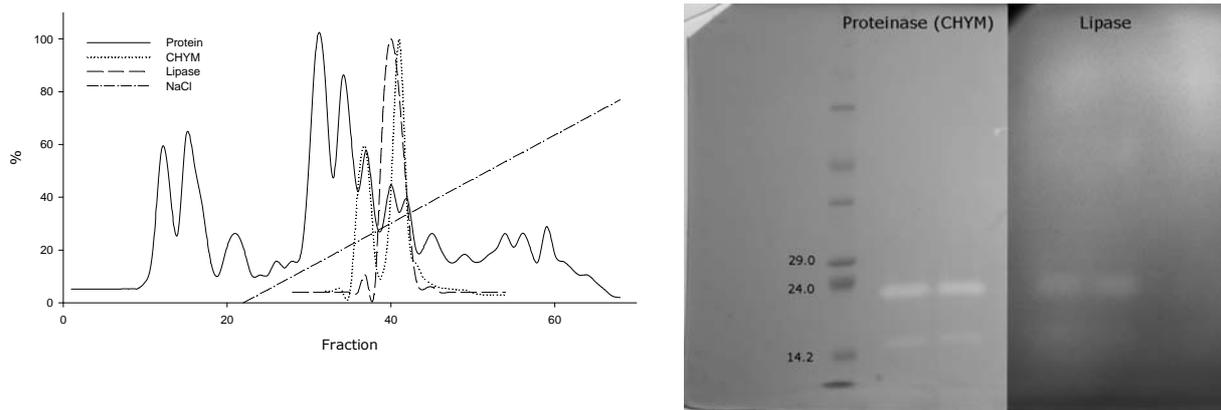


Fig. 12  Purification of *X. testudinatus* chymotrypsins (CHYM) by anionic exchange chromatography. Fig (a) represents the complete elution profile of mid gut gland extracts and the second purification step of CHYM (dashed line) and a further enzyme with lipolytic activity (dotted line). Chymotrypsin activity was assayed using SAAPNA and lipase activity with MUF-butyrate (see material and method part). The column was equilibrated with buffer A (0.01 mol·l$^{-1}$ imidazole buffer, pH 6.8), and afterwards enzymes were eluted with a linear gradient of 0-100% buffer B (0.01 mol·l$^{-1}$ imidazole buffer (pH 6.8) with 1 mol·l$^{-1}$ NaCl). (b) SDS-Page performed on a single gel indicating the presence of CHYM and lipase activity in purified samples.
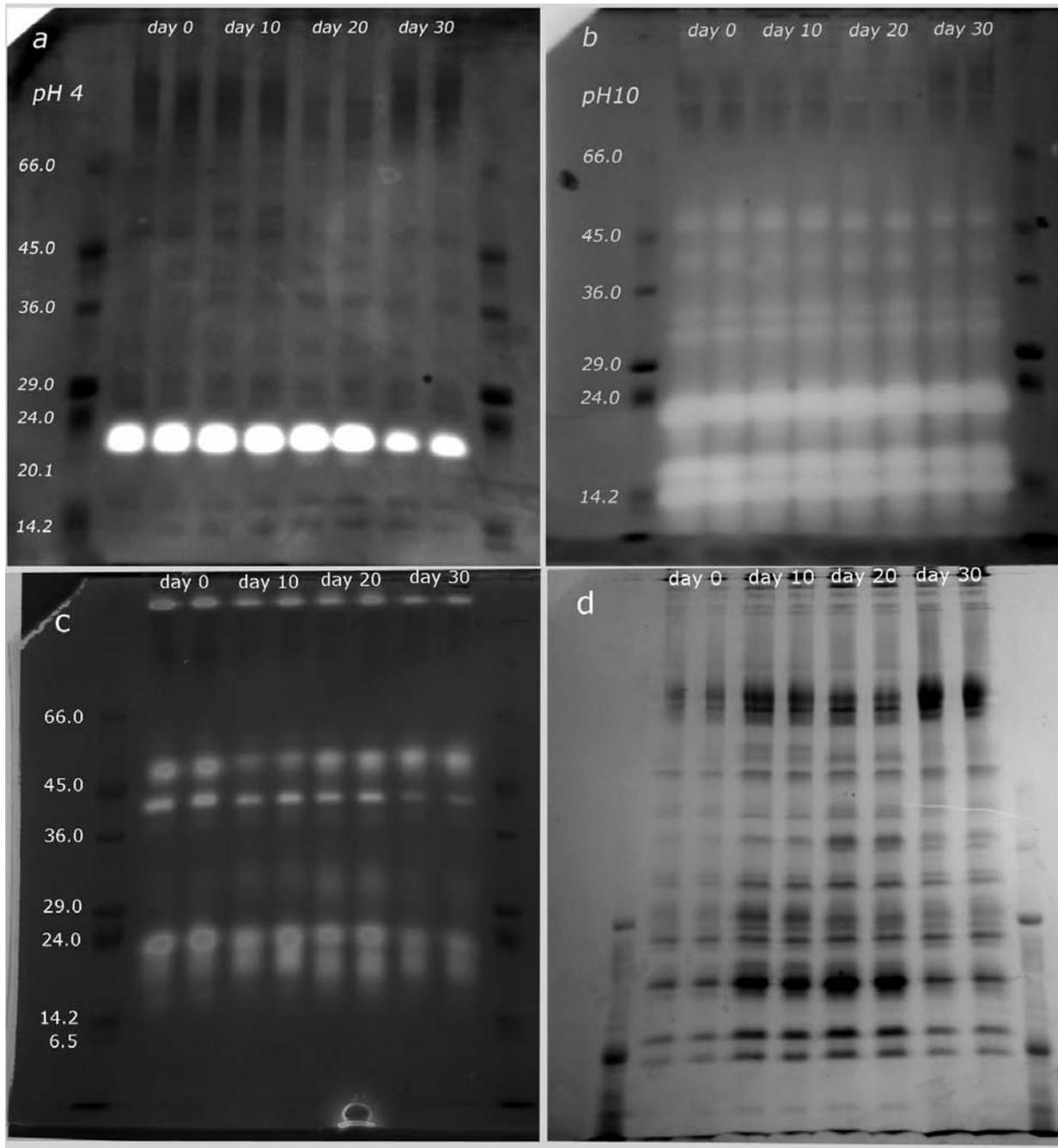
Fig. 13 Zymograms for proteolytic and lipolytic activities along the starvation period. Comparison of proteolytic activities at acidic and alkaline pH (a + b) investigated by using Haemoglobin or Casein solved in universal buffer as substrate. Zymograms of lipolytic activity at pH 7 (c) and total proteins after coomassie staining (d). Molecular weights are given in kDa.

*3.4.4. Effects of starvation on lipolytic and proteolytic activities*

Starvation for 30 days caused a significant decrease of proteolytic as well as lipolytic activity (Fig. 14). First the loss of total proteolytic activity was low but became significant (60 to 70 % residual activity) after 20 and 30 days. The course of lipase/esterase activity during starvation was similar but the decrease in activity was much stronger. After ten days of starvation the residual lipolytic activity decreased to less than 60% and after 30 days to about 30% of initial activity.
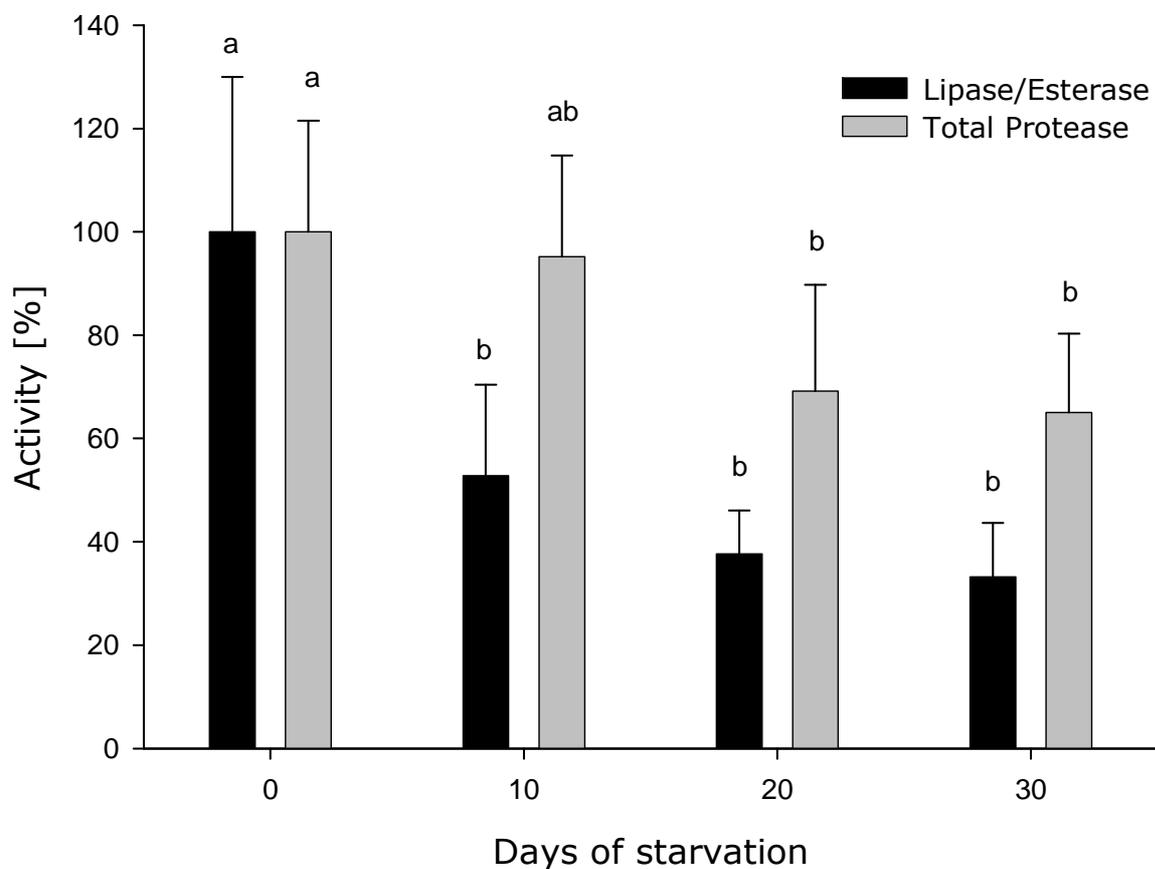


Fig. 14 Comparative plot of changes in the relative activity between Lipase/Esterase and Total Protease activity in fed and starved animals (n=7). During starvation a higher decrease in lipolytic activity can be observed compared to the decease in proteolytic activity. Different letters within one enzyme denote significant changes of activity during starvation.

*3.5. Lipids*

The total lipid contents of the midgut glands ranged from 53.8 ± 4.2 % to 36.8 ± 16.3 % of DM. (Fig. 15). Highest lipid values were found in freshly sampled animals but declined to 43.9 ± 11.6 % after ten days and to 40.9 ± 10.4 % after twenty days of starvation. The minimum of 36.8 ± 16.3 % was reached after 30 days of starvation. Due to high variation the differences in lipid contents were not statistically between sampling days.
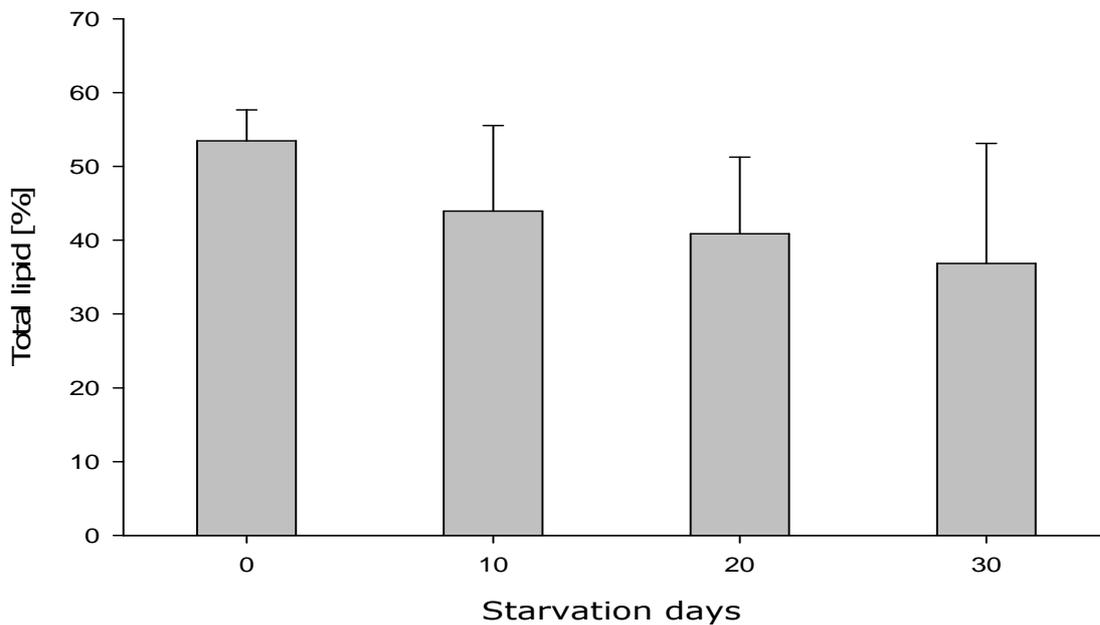


Fig. 15  Lipid contents of *X. testudinatus* mid gut glands after starvation for 0, 10, 20 and 30 days. Values are given in % of mid gut gland dry mass (DM). Bars indicate standard deviations (n = 9-10).

The majority of lipids were triacylglycerols (TAG, neutral lipids) and polar lipids (PL) which together accounted for 95% of total lipids (TL) in fed and in starved animals (Fig. 16). After starvation for 30 days the amount of TAG declined from 92.0±2.5% TL to 76.3±23.5% TL. Simultaneously, the share of PL increased from 4.0±2.2% TL to 17.4±19.6% TL. These results clearly show a decrease of TAGs during starvation but due to increasing inhomogeneity among starved animals the differences between fed and starved specimens were not statistically significant. Free fatty acids (FFA) as well as sterols (ST) contribute only little to the total lipid content.
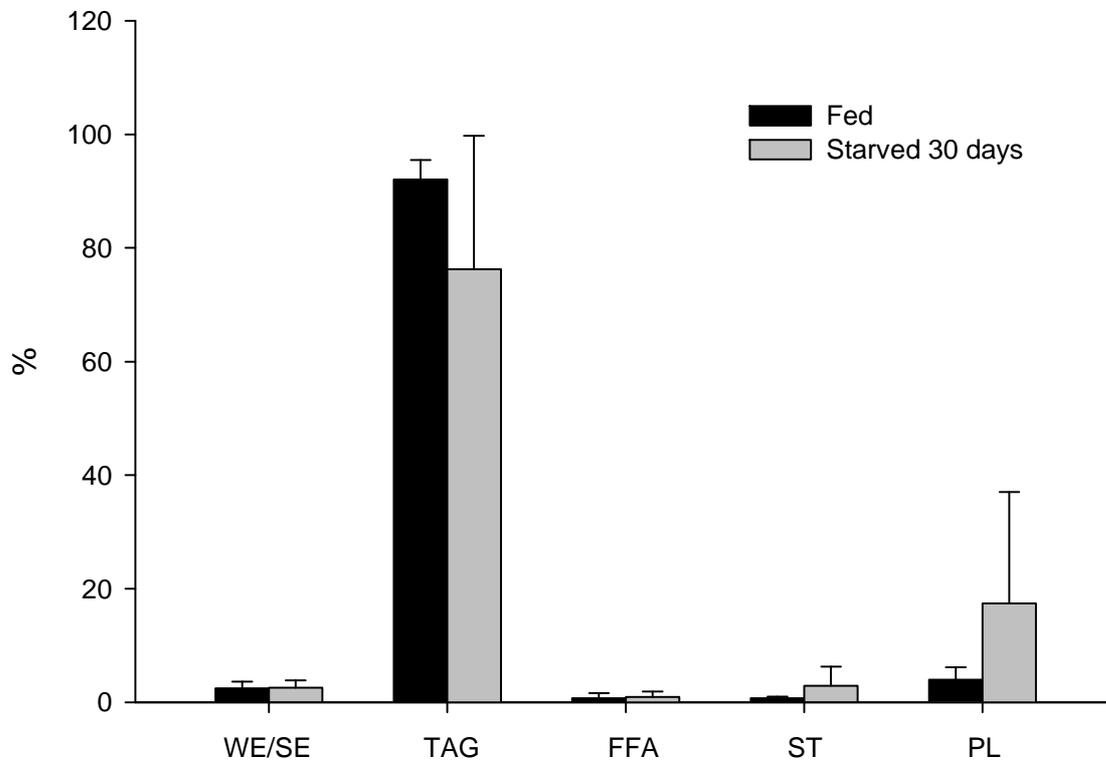
Fig. 16 Lipid classes (LC) in % of total lipid (TL) of *X. testudinatus* midgut glands showing triacylglycerols (TAGs), polar lipids (PL) and, wax-/Sterol-esters. Bars in indicate standard deviations (Fed n=10; starved 30 days n=9)

The fatty acid composition of fed and starved specimens was dominated by saturated (SFA) and monounsaturated fatty acids (MUFA) while only few polyunsaturated fatty acids (PUFA) were present (Fig. 17). The major FA was the MUFA 18:1(*n*-7) with 22.8±2.3% (fed) and 24.7±4.6% (starved 30 days) of total FA. Other MUFAs were 16:1(*n*-7) (~10%) and 18:1(*n*-9) (~12%). The SFA 18:0 amounted to about 5% and 16:0 to ~17% of total FA. . The major PUFAs, 22:5(*n*-3) and 22:6(*n*-3), contributed together only 11% of total FA. During the 30 days of starvation no statistically significant changes of FA patterns was evident. Only 16:1(*n*-7) decreased significantly after 30 days starvation.

Table 4  Mid gut gland fatty acids of *X. testudinatus*

| Fatty acids | Starvation (day 0) Mean ± S.D. (10) | Starvation (day 10) Mean ± S.D. (10) | Starvation (day 20) Mean ± S.D. (10) | Starvation (day 30) Mean ± S.D. (9) |
|---|---|---|---|---|
| 14:0 | 1.1 ± 0.3 | 2.0 ± 0.7 | 2.3 ± 0.4 | 0.7 ± 0.3 |
| 14:1($n$-5) | 0.1 ± 0.1 | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.0 ± 0.1 |
| 15:0 | 0.3 ± 0.1 | 0.4 ± 0.2 | 0.5 ± 0.1 | 0.3 ± 0.2 |
| 14:0A | 0.0 ± 0.1 | 0.3 ± 0.2 | 0.3 ± 0.1 | 0.1 ± 0.1 |
| 16:0 | 16.5 ± 5.8 | 18.8 ± 3.3 | 20.4 ± 0.8 | 18.0 ± 3.2 |
| 16:1($n$-7) | 10.8 ± 1.2 | 11.1 ± 1.8 | 11.4 ± 1.9 | 8.3 ± 1.7 |
| 16:1($n$-5) | 0.5 ± 0.1 | 0.7 ± 0.2 | 0.6 ± 0.1 | 0.5 ± 0.1 |
| 16:2($n$-4) | 0.6 ± 0.2 | 0.5 ± 0.2 | 0.5 ± 0.2 | 0.6 ± 0.1 |
| 17:0 | 0.5 ± 0.1 | 0.5 ± 0.1 | 0.5 ± 0.1 | 0.6 ± 0.2 |
| 16:3($n$-4) | 0.4 ± 0.1 | 0.4 ± 0.2 | 0.4 ± 0.1 | 0.4 ± 0.2 |
| 16:0A | 0.1 ± 0.1 | 0.3 ± 0.2 | 0.4 ± 0.2 | 0.3 ± 0.1 |
| 18:0 | 5.2 ± 0.6 | 5.1 ± 1.0 | 5.0 ± 0.6 | 5.8 ± 0.7 |
| 18:1($n$-9) | 11.3 ± 1.7 | 13.0 ± 3.0 | 11.4 ± 2.3 | 11.8 ± 0.9 |
| 18:1($n$-7) | 22.8 ± 2.2 | 18.9 ± 6.4 | 21.7 ± 3.4 | 24.6 ± 4.7 |
| 18:1($n$-5) | 0.4 ± 0.1 | 0.4 ± 0.1 | 0.4 ± 0.0 | 0.5 ± 0.2 |
| 18:2($n$-4) | 0.4 ± 0.1 | 0.3 ± 0.1 | 0.4 ± 0.0 | 0.3 ± 0.1 |
| 18:2($n$-6) | 1.1 ± 0.3 | 1.4 ± 0.7 | 1.0 ± 0.4 | 1.1 ± 0.4 |
| 18:3($n$-6) | 0.3 ± 0.1 | 0.1 ± 0.2 | 0.1 ± 0.1 | 0.3 ± 0.1 |
| 18:3($n$-3) | 0.6 ± 0.1 | 0.7 ± 0.2 | 0.6 ± 0.2 | 0.5 ± 0.2 |
| 18:4($n$-3) | 0.5 ± 0.3 | 0.2 ± 0.3 | 0.0 ± 0.0 | 0.2 ± 0.2 |
| 20:0 | 0.4 ± 0.1 | 0.5 ± 0.2 | 0.0 ± 0.0 | 0.5 ± 0.1 |
| 20:1($n$-11) | 0.4 ± 0.0 | 0.4 ± 0.1 | 0.2 ± 0.2 | 0.4 ± 0.2 |
| 20:1($n$-9) | 0.9 ± 0.2 | 0.9 ± 0.4 | 0.7 ± 0.5 | 1.0 ± 0.2 |
| 20:1($n$-7) | 2.1 ± 0.4 | 1.9 ± 0.8 | 2.1 ± 0.4 | 2.7 ± 0.9 |
| 20:2($n$-6) | 0.4 ± 0.3 | 0.3 ± 0.2 | 0.3 ± 0.1 | 0.4 ± 0.2 |
| 20:3($n$-6) | 0.2 ± 0.1 | 0.2 ± 0.2 | 0.1 ± 0.2 | 0.3 ± 0.1 |
| 20:4($n$-6) | 2.0 ± 0.5 | 2.4 ± 1.7 | 1.9 ± 0.8 | 2.6 ± 1.1 |
| 20:4($n$-3) | 0.4 ± 0.1 | 0.4 ± 0.2 | 0.3 ± 0.2 | 0.2 ± 0.1 |
| 20:5($n$-3) | 4.9 ± 1.1 | 4.7 ± 1.8 | 4.2 ± 1.0 | 4.7 ± 1.5 |
| 22:0 | 0.4 ± 0.1 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.5 ± 0.1 |
| 22:1($n$-11) | 0.5 ± 0.1 | 0.6 ± 0.4 | 0.5 ± 0.2 | 0.5 ± 0.2 |
| 22:5($n$-3) | 0.8 ± 0.2 | 0.7 ± 0.3 | 0.6 ± 0.2 | 0.6 ± 0.3 |
| 22:6($n$-3) | 7.7 ± 1.7 | 6.4 ± 2.6 | 5.9 ± 1.7 | 6.3 ± 2.2 |
| unknown | 4.1 ± 2.5 | 4.7 ± 3.4 | 3.3 ± 0.8 | 3.6 ± 1.4 |
| | | | | |
| Sum SAT | 24.4 ± 7.0 | 27.3 ± 5.4 | 28.7 ± 2.0 | 26.4 ± 4.8 |
| Sum MUFA | 49.9 ± 6.2 | 48.1 ± 13.4 | 49.3 ± 9.3 | 50.3 ± 9.1 |
| Sum PUFA | 20.5 ± 5.3 | 18.4 ± 8.9 | 16.4 ± 5.3 | 18.4 ± 6.6 |
| Sum ($n$-7) | 35.7 ± 3.8 | 32.0 ± 8.9 | 35.3 ± 5.7 | 35.6 ± 7.3 |
| 18:1(n-7)/18:1(n-9) | 2.0 | 1.5 | 1.9 | 2.1 |

Fatty acid composition (mass% of total fatty acids) of starved (0, 10, 20, 30 days) specimens presented as mean and standard deviation (S.D.); SAT (saturated fatty acids), MUFA (monounsaturated fatty acids), PUFA (polyunsaturated fatty acids) and ($n$-7) (fatty acids from the ($n$-7) series); (n) Number of specimens tested.

Fig. 17 Composition of major fatty acids (> 4.5%) of the mid gut glands from freshly caught and 30 days starved *X. testudinatus*. Fatty acids <4.5% were summed up and presented as single bar. The FA amounts are given as per cent values of total fatty acids. Bars indicate standard deviations (n = 9-10). Significant differences are indicated by asterisk (Students t-test; ** $p<0.01$).

## 4. Discussion

### *4.1. Foregut morphology*

Although relatively rich information about foregut ossicles in shrimps exists, only little is reported on crab foregut morphology. Major studies in this field are given by Mocquard (1883) who described the stomach morphology of more than 60 species, Ceccaldi (1989) who described the physiological and morphological digestive features of decapods reared in aquaculture and Martin et al. (1998), who compared foregut morphologies of deep sea hydrothermal vent brachyurans. All these studies indicate a similar basal morphology of brachyuran major foregut ossicles including one median tooth, a pair of lateral teeth and accessory teeth and the cardiopyloric valve. One main focus in studies of crab foregut morphology was dedicated to the question in which degree the morphology reflects ancestry over adaptation effects, vice versa (Caine 1975; Felgenhauer and Abele 1983). Unfortunately, the existing information of foregut morphologies of other hydrothermal vent crabs is very scarce due to the fact, that these environments are seldom and not easily accessible. In this context the work of Martin et al. (1998), has to be mentioned, which investigates the major foregut ossicles morphology of deep sea hydrothermal vent crabs in a comparative study. Comparisons of *X. testudinatus* foregut ossicles with those of other Brachyuran crabs (Meiss and Norman 1977) and of the deep sea hydrothermal vent crab *Bythograea thermydron* underline the observation of similar general morphological features within brachyuran crabs, but reveal no detailed similarities due to their comparable habitats. The median tooth of both deep sea hydrothermal vent species is surrounded by a plate whose margins are serrate or scalloped, and which has spinulose setae on the tip of each serration (Martin et al. 1998). The lateral teeth of this species look like mill plates and remind of a "cattle jaw" designed to grind delicate material. In contrast to letter, the lateral teeth of *X. testudinatus* are designed to cut ingested tissues with blade shaped teeth. The shape of the median tooth of *X. testudinatus*, who belongs to the grapsidae, shows many similarities to that of *Pachygrapsus crassipes*, a "typical" grapsid crab also described (Martin et al. 1998).

*4.2. Metabolic rates*

The general characteristic of *X. testudinatus* oxygen consumption was an increase of respiration with rising temperatures. This observation can be considered as typical for poikilotherm organisms and results are in accordance with those obtained from other invertebrates (Ali et al. 2000; Taylor and Peck 2004; Kunzmann et al. 2007). The magnitude of increase in oxygen uptake due to rising ambient temperatures follows the van t `Hoff`s law in the temperature range between 20 and 30°C with $Q_{10}$-values around 2 similar to those found in other crustaceans (Saborowski et al. 2000; Taylor and Peck 2004). One possibility why $Q_{10}$-values below 20°C do not follow an exponential function, are temperature adaptation effects. Cold adapted organisms like the northern krill, *Crangon* spp. or deep sea species show constant metabolic rates at rather low temperatures (Saborowski et al. 2000; Chausson et al. 2004) whereas tropical species are adapted to higher temperatures. Survival rates clearly represent the optimum temperature range of an organism (Haefner 1969). If a tropical species like *X. testudinatus* is exposed to unusual low temperatures, as it is the case with 15°C, the metabolism is out of adaptation range and finally cannot function efficiently enough to keep the animal alive. This phenomenon can be underlined by lab observations in which crabs stopped feeding at temperatures below 15°C. The fact that hermatypic corals are located in similar depth on the western end of Kueishan Island support the assumption of constant temperatures between 20°C and 30°C in this area. Furthermore, oxygen consumption rates provide information about energetic demands which demonstrated that one individual of 10 g body weight needs 326 Joule or 78 calories per day (25°C) during routine activity. Calculations made according to the information that 1 g of ash-free dry weight of copepods contain between ~22-31 kJ reveal a necessary daily zooplankton (copepods) uptake of 100 mg (Fresh weight; and assuming a mean water content of 90%) in order to keep basic energy demands (Salonen et al. 1976). These results are consistent with findings made by Taylor and Peck (2004) who determined daily energy demands of the sand shrimp, *Crangon sptemspinosa*. Large individuals (1.5 g FW) of these shrimp have a daily energy demand of 300-400 J, at an ambient temperature of 20°C. A relatively lower result for *X. testudinatus* may be explained by the more sluggish lifestyle and the increased body mass caused by their more solid shell compared to those of shrimps.

The ability of *X. testudinatus* to survive in this adverse and food scarce environment is mainly based on the specialized and synchronized feeding behaviour during the short period of slack water, observed by Jeng et al, (2004). During this time the animals are forced to feed as much as possible in order to overcome longer starvation periods. In respect to this knowledge, the physiological adaptations allowing fast and effective food uptake and energy storage can be assumed as a key feature for the survival of this species.

*4.3. Digestive features*

*X. testudinatus* mid gut gland extracts represent a wide range of digestive abilities, with several dominating activities among all groups of digestive enzymes (e.g. Peptidases, Lipases, Glucosidases and Phosphorylases. The presence of unspecialized digestive abilities, underline the heterotroph, and non-selective feeding behavior of this species, mainly scavenging on killed plankton "marine snow" that sinks down to the sea-floor. Due to the observation of large bacterial mats in some areas, and on the bodies of the crabs the question for a bacterial food source was raised. It is well established that cell wall cleaving enzymes like lysozyme or N-acetyl-β-glucosaminidase indicate bacteria digestion (Boetius and Felbeck 1995). The mid gut gland extracts revealed no lysozyme, but rather high N-acetyl-β-glucosaminidase (chitobiase) activities. As crustaceans rely on a chitobiase when feeding on other crustaceans, or during moulting (Oosterhuis et al. 2000), the presence of this enzyme itself, can not serve as an indicator for the utilization of bacteria. If, in the case of this species, chitobiase is additionally used for digestion of bacteria one could rather assume an opportunistic feeding on bacteria.

Further, a main focus was dedicated to the total proteolytic activity and the characterization of the serine proteases, trypsin and chymotrypsin. The activities of trypsine, measured at 30°C were $9.15\pm3.32$ [U·min$^{-1}$·g$_{FM}$$^{-1}$] and $68.28\pm11.10$ [U·min$^{-1}$·g$_{FM}$$^{-1}$] for chymotrypsin, and can be considered as very high compared to other crustaceans. Teschke and Saborowski (2005) demonstrated that the southern krill, *Euphausia superba* can be characterized by considerably high ($6.6\pm2.0$ [U·min$^{-1}$·g$_{FM}$$^{-1}$]) trypsin capacities compared to crustaceans like the hermit crab *Pagurus bernhardus* ($3.4\pm0.7$ [U·min$^{-1}$·g$_{FM}$$^{-1}$]), *Cancer pagurus* ($3.3\pm0.6$ [U·min$^{-1}$·g$_{FM}$$^{-1}$]) or *Pandalus montagui* ($1.3\pm0.4$ [U/min·g$_{FM}$]). The high digestive capacities of krill can be explained by their feeding behaviour on patchy food sources (Morris et al. 1983; Buchholz and Saborowski 2000). Once a swarm of krill hits into a food patch, they can rapidly digest, all necessary food

items. Under this light, highly efficient digestive capacities of krill can be considered as one of the central features to survive in polar waters. Trypsin activities measured for *X. testudinatus* were at least as strong as those determined for *Euphausia superba* by Teschke and Saborowski under same conditions and with the same substrate. These comparable proteolytic features can be explained by similar feeding strategies. Like the Antarctic krill, *X. testudinatus* feeds on a high concentrated food source which occurs just during slack water, when zooplankton killed by the toxic discharges of the vents, rains down like "marine snow". During this moment the crabs swarm out of their cervices and start to feed rapidly, in order to avoid gratuitous exposure to the toxic environment and to make use of the scarce food to full capacity.

Although physiological response towards a specialized feeding behavior was demonstrated, the question regarding enzymological adaptations towards an extreme environment and the exposure to toxins by ingested food still needs to be illuminated in the following. Due to high ambient water temperatures in hydrothermal vent habitats (McMullin et al. 2000), evolutionary adaptations on the protein/enzyme level could be regarded as essential features to assure survival. Although hot water discharged by the vents merges quickly with the surrounding seawater, elevated temperatures can be expected due to short time exposures, or the shallow andestic lava flows which heat up the seafloor. The results of the present study demonstrate that *X. testudinatus* total proteinase tolerates temperature exposures to 55°C for 5 h without noticeable loss of activity. The trypsin-like proteinase of this species show remarkable stability and remain unimpaired even after exposures to 60°C for a 5 h incubation period. Compared to similar investigations (Dittrich 1992a; Dittrich 1992b; Garcia-Carreno and Haard 1993; Garciacarreno and Haard 1993; Oh et al. 2000) *X. testudinatus* proteinases show elevated thermal tolerance. The study by Dittrich (1992b) compared trypsin-like proteases of crustaceans from tropical, temperate and subarctic regions. In this work thermal stability of trypsin was investigated under same conditions as in the present study and revealed lowest thermal stabilities for polar species like the copepod *Calanus acutus* (loss of > 50%, 2 h at 40°C) or the shrimp *Chorismus antarcticus* (loss of >90%, 2 h at 40°C), whereas tropical species like the hermit crab *Clibanarius striolatus* and *Ocipode ryderi* showed thermal stabilities along 2 h to 40°C and 50°C. At 60°C residual activities for both tropical species decreased to 0% after 30 min and 90 min. Regarding the fact that the hydrothermal vent crab *X. testudinatus* exhibits trypsin-like proteases which function at elevated temperatures of 60-65°C and total proteinase which functions to 55°C for 5 h without loss of activity, these enzymological features, in terms of high stability, can be regarded as adaptations towards a

habitat exhibiting elevated temperatures and various enzyme inhibiting compounds. In contrast to the increased thermal stability, the temperature optima of proteases reveal no elevated working temperature. The working optimum of the serine proteases Trypsin (60°C) and Chymotrypsine (50°C) show no distinctive differences from other tropical crustaceans (Dittrich 1992b; Pavasovic et al. 2004).

The proteases Trypsin and Chymotrypsin, which belong to the family of serine proteases can be considered as the most intensively investigated, and probably the most important digestive enzymes present in crustaceans (Tsai et al. 1991; Klimova and Chebotarev 2000; Pavasovic et al. 2004). In the course of enzyme characterizations, the pH working optimum of these enzymes has been frequently investigated (Garcia-Carreno and Haard 1993; Garciacarreno and Haard 1993; Hernandez-Cortes et al. 1997; Del Toro et al. 2006), revealing the slightly alkaline working optima for serine proteases. Dionysius et al. (1993) reported a pH optimum of 8 for a Trypsin-like protease isolated from the sand crab *Portunus pelagicus*. Similar results with highest activities at pH 7.5-8 were observed for Trypsins of *Paralithodes camchatica* (Rudenskaya et al. 2000). The results of this study demonstrated that both, Trypsin and Chymotrypsin of this hydrothermal vent species show a wide pH-stability range from acidic to alkaline pH (pH 6-10). In respect to the fact, that the hydrothermal vent habitat of Kueishan Island is characterized by highly acidic discharges, the enzymatic tolerance for a wide pH-range can be considered as an essential feature of this crab. The hurried and non selective feeding of *X. testudinatus* during the short slack water period described by Jeng et al. (2004), prohibit a proper selection of food particles. As a consequence, digestive enzymes might be short-term exposed to ingested, food particles or water with extreme pH, which are later on adjusted to the internal gastric pH. In order to resist these pH variations a wide pH-stability range can be regarded as an important feature.

The present study shows, that Chymotrypsin has its working optima at pH 8 and Trypsin even at pH 10. Under the beforehand mentioned circumstances, of acidic environmental conditions, an alkaline working optimum for major digestive enzymes, appears to be contradictory. Regarding the question, why Trypsins of *X. testudinatus* showed working optima at much higher pH (pH 10), compared to other crustaceans, two explanations can be proposed.

1. Neutralization: If enzymological features can be regarded as adaptations to an elevated internal pH, this alkaline internal pH can be assumed to function as a neutralization mechanism.

Ingested food or water with generally low pH can be immediately neutralized by internal alkaline conditions.

2. Detoxification: Alkaline conditions favour HS⁻ over the more toxic $H_2S$.

In consideration of high sulphide concentrations in this volcanic active habitat, the aspect of detoxification, e.g. sulphide-tolerance, can be regarded as an essential feature for survival. The mechanism to avoid $H_2S$, which is believed to be the more toxic form of sulphide, by elevated internal pH has been reported from the tubeworm *R. pachyptila.* In-vivo experiments demonstrated, that the internal alkaline pH of *R. pachyptila* is tightly regulated, despite changing internal $CO_2$ and $H_2S$ concentrations (Goffredi et al. 1997). In the case of these deep-sea tubeworms, the elimination of proton-equivalents is managed by active transport mechanisms which correlate with the oxidation of sulphide by endosymbiontic bacteria (Girguis et al. 2002). For *X. testudinatus*, who shows high sulfide tolerances, the mechanisms of detoxification are still poorly understood.

Based on the fact, that vent habitats show high concentrations of metal ions, such as $Mg^{2+}$, $Mn^{2+}$,$Fe^{2+}$, $Cu^{2+}$ and $Ca^{2+}$, which are well known for their inhibitory effects on enzymatic processes, the question about the resistance of digestive enzymes against these inhibitors displays an important issue (Dreyfus and Iglewski 1986; Edgcomb et al. 2004; Cardigos et al. 2005). The results of this work corroborate with earlier findings, which demonstrated that metal ions like $Fe^{2+}$, $Cu^{2+}$ inhibit enzyme activities (Sakharov and Prieto 2000). For the inhibition experiments all inorganic inhibitors were adjusted to 10 mM concentrations. Compared to dissolved metal ion concentrations reported for hydrothermal vent habitats, which are usually in the sub-micro to nano molar range, $0.01 \text{ mol·l}^{-1}$ can be considered as an extremely high and a lethal dosage for aquatic organisms (Olaifa et al. 2004; Cardigos et al. 2005). Although excessive concentrations were applied in this test, the activities of Trypsin and Chymotrypsin were not completely inhibited, still allowing proteolytic activity at low levels. Furthermore, the strongly elevated proteolytic activity of *X. testudinatus* might support the digestive capacity in the presence of inhibitors.

In order to receive a broad overview of the proteolytic features of this species, SDS-Page assays were performed. The results revealed the presence of at least 7 proteases, active at alkaline pH (pH 10) and only one acidic protease active at pH 4. These findings once again underline the

general favouring of alkaline internal conditions over acidic, by the proteolytic enzymes. The only active protein band present at acidic pH (below 24 kD) also appears under alkaline conditions indicating the existence of a protease, active under a wide pH range. Klimova and Chebotarev (2000) characterized the collagenolytic proteases of the Kamchatka crab, *Paralithodes camtschatica*, and demonstrated the presence of 9 protease of which 7 exhibited azocasein-hydrolysing ability. Among these, a 25 kD protease also showed a wide pH range, peaking at pH 7.5 and with low residual activities at pH 12 and 3. The presence of acidic proteases, mainly belonging to the group of aspartic proteases, was reported for several crustaceans, including *Homarus gammarus*, *Panulirus interruptus* and *Cancer pagurus* (Del Toro et al. 2006). Additionally performed lipase activity gels showed the presence of at least 4 activity bands, whereupon the upper most one can be assumed to consist of membrane-bound lipases, still attached to membrane fragments. However, the strongest lipase band was located at the same position as the wide pH-range protease described above. The results of the ion exchange chromatography strongly suggest, that it concerns of one enzyme with both, lipase and Protease/Collagenase activity. Existence of such multifunctional enzymes are poorly documented, and thus displaying an interesting aspects for further investigations.

The digestive enzyme responses of *X. testudinatus* to starvation showed a significant decrease for both, lipase and protease activity along the starvation period of 30 days. This observation corroborates with findings made not only in crustaceans, but also in many other phyla (Fisk and Shambaugh; Ceccaldi 1989). From an energetic point of view, a reduction of digestive enzyme synthesis displays a reasonable reaction towards starvation. However, it has been documented that the course of activities during starvation differs among specific enzymes, indicating fuelling of different energy sources (Johnston et al. 2004). This phenomenon is also visible in *X. testudinatus*, where proteases decrease much slower and maintain higher activities during starvation than lipases. This maintenance of higher proteolytic capacities can be explained by continuing catabolic processes preferably utilizing protein reserves, especially in the initial starvation phase of *X. testudinatus*.

*4.4. Energy household*

In respect to the beforehand mentioned high digestive activity, which results with the utmost probability from an irregular and patchy food availability, a further necessary physiological feature for the successful survival of this species was assumed: Energy storage due to prolonged starvation periods. The determination of total lipid in the mid gut gland of *X. testudinatus* revealed high amounts of lipids (~50% of mid-gut gland dry mass) in this organ. It is well established that the mid gut gland of crustaceans functions as the main storage organ for energy equivalents, e.g. lipids (O'Connor and Gilbert 1968; Lawrence 1976). High amounts of lipid were mainly reported for polar to temperate species that have to cope with long starvation periods and tightly regulated reproduction periods due to seasonal changing conditions (Hagen et al. 1996; Styrishave and Andersen 2000). In contrast, there is no necessity for tropical epipelagic species to store lipids because of constant primary productivity, and consequently food supply over the whole year (Lee and Hirota 1973; Lawrence 1976). The question why *X. testudinatus* stores high amounts of lipids can be answered by the habitat these animals are living in. Due to the extreme toxic discharges of the vents, this habitat is inhospitable for most organisms, and can be regarded as separated from the surrounding environment. The only input of particular organic matter (POM) consists of organisms killed by the toxic discharges, and sinking to the seafloor. As these crabs mainly rely on this food source, whose availability is strongly influenced by tides and local currents, the ability to overcome periods of food deprivation can be regarded as an essential feature. Starvation experiments conducted in this thesis underline the proposed ability to overcome prolonged starvation periods. The majority (>90%) of animals survived, at least, 30 days without feeding.  The highest mortality along this starvation experiment was observed during the first two weeks, after capture and transfer to the testing setup, indicating rather stress-over starvation- dependent mortality. Similar results were reported for the mitten handed crab *Eriocheir sinensis* that showed the ability to overcome starvation periods as long as 70 days (Wen et al. 2006). The findings made by Wen et al., (2006) demonstrated, that the mitten handed crab, an almost ubiquitous species present in tropical as well as temperate regions, stores significant amounts of lipids in the mid gut gland. Due to the fact, that both, *E. sinensis* and *X. testudinatus*, show comparable amounts of lipid reserves in relation to their body mass, one can assumed that the starvation limit of *X. testudinatus* was not jet reached. In respect to daily energy demands of 326 J and an average lipid content of 74.7 mg for a 10 g individual, the remaining time can be

calculated until all energy reserves will be used up during starvation. According to the generally accepted average physical energetic equivalents for lipids (39.7 kJ·g$^{-1}$) (Salonen et al. 1976) the lipid reserves in the mid gut gland may be sufficient for only nine days. The fact that *X. testudinatus* survived at least 30 days without food, indicates metabolic adaptations along the course of starvation, or alternative energy sources. It is well established that crustaceans respond to starvation with decrease of metabolic rates (Wallace 1973; Hervant et al. 1999). The reduction of metabolic rates during starvation, reported for the common shore crab *Carcinus maenas* were as high as 40-50% during a three month starvation experiment made by Wallace (1973). Regarding this information, the energy reserves of *X. testudinatus* may last for 20 days, but never for more than 30 days. During starvation the chemical body composition changes and body tissues are lost due to catabolic activities (reviewed by Stuck et al. 1996). It was shown, that besides lipid reserves, alternative metabolic stores like proteins and glycogen can serve as energy sources in crustaceans (Oliveira et al. 2004). The importance and the order of utilization varies among species. Some species respond to starvation by utilizing carbohydrate stores (mainly glycogen) first, followed by lipid reserves and finally proteins (Stuck et al. 1996; Oliveira et al. 2004). In other species glycogen plays a less important role during food deprivation, consequently the predominant energy reserves consists of lipids or proteins, or both (Hagen et al. 1996; Rosas et al. 2002; Wen et al. 2006; Sánchez-Paz et al. 2007). Findings made by Hervant et al. (1999) showed a preferred metabolization of proteins over lipids in subterranean and surface dwelling amphipods. This phenomenon would generally explain a rather low decrease in lipid stores, associated with higher proteolytic activities than lipolytic activities in the mid gut gland. The observations made in the present study indicate a possible interaction of several physiological responses to starvation, triggering the metabolic energy-saving mechanisms. An answer to these questions is essential for the understanding, how this hydrothermal vent crab copes with prolonged periods of food deprivation.

Neutral lipids of *X. testudinatus* were predominantly stored as TAG. The high amount and their decline during starvation, underlines the necessity of high energy reserves due to sporadic food availability. Besides the dominant TAGs, midgut glands contained 4% PL and 2.5% WE/SE. TAG are short-term, whereas WS are long-term energy reserves  The occurrence of WE was not an isolated event, but appeared in every individual tested. Although these compounds appeared only in small amounts, their presence was continuous and not starvation dependent. This observation indicates a real storage rather than an artefact by ingested WE that would have been

excreted during 30 days. The occurrence of similar amounts of WE was reported for the hydrothermal vent crab *Bythograea thermydron*. This species showed low amounts of WE in adult specimens, but rather high amounts in juveniles (16%) (Phleger et al. 2005b). Similar observations were made for hydrothermal vent shrimps (*Alvinocaris markensis* and *Rimcaris exoculata*) showing high WE in zoea and early postlarvae and no WE in adults (Pond et al. 1997b). Accordingly, the presence of WE in *Xenograpsus* adults suggests a similar analogue ontogenetic shift in utilizing WE. High energy reserve in deep sea hydrothermal vent crustacean larvae indicate a bathypelagic lifestyle enabling them to maintain a prolonged planktonic existence, and thus a wide spread dispersal in their search for new vents (Pond et al. 1997b). In the case of the *X. testudinatus*, an endemic species a Kueishan island a wide dispersal may not be an advantage and was not observed to this state. It can be assumed, that food for *X. testudinatus* larvae is almost non existent, because of the food scarce environment, and consequently affords high energy reserves for their development. However, the physiological properties of larval stages remain unknown and thus represent an interesting aspect for further investigations.

The mid gut fatty acid composition of *Xenograpsus* adults is dominated by MUFAs of the (*n*-7) and (*n*-9) series and the SFA 16:0, but PUFAs like 20:5(*n*-3) (EPA) and 22:6(*n*-3) (DHA) are also existent. These long chain PUFAs are regarded as essential FA which can not be biosynthesized by heterotrophic organisms (Dalsgaard et al. 2003). These PUFAs from the (*n*-3) series commonly derive from plants, but also from few invertebrates and protozoa (Pond et al. 1997a; Pond et al. 2002). High amounts of the MUFA 18:1(*n*-9) underlines the rather carnivorous feeding described by Jeng et al. (2004). 18:1(*n*-9) displays the major FA in most marine animals and thus it is generally accepted as marker for carnivory (Dalsgaard et al. 2003). Furthermore, the ratio 18:1(*n*-7)/18:1(*n*-9) is also used as an indicator for carnivorous feeding based on higher uptake of 18:1(*n*-9) with zooplankton. Consequently this ratio increases during starvation, due to a loss of 18:1(*n*-9), and thus can not serve as an unambiguous indicator for carnivorous or herbivorous feeding. This increase of the 18:1(*n*-7)/18:1(*n*-9) ratio is visible in starvation levels of *X. testudinatus* and corroborates with previous findings indicating a utilization of TAGs during food depletion.

Deep sea vent habitats can be regarded as enclosed oasis of life based on a bacterial primary production. Consequently all trophic levels are more or less characterized by bacterial fatty acid trophic markers, providing a fingerprint for deep sea hydrothermal vent organisms (Pond et al. 1998; Dalsgaard et al. 2003; Phleger et al. 2005a; Phleger et al. 2005b). It is generally accepted,

that fatty acids belonging to the MUFA ($n$-7) series are predominantly produced by phytoplankton and bacteria and not by animals (reviewed by Dalsgaard et al. 2003). As deep sea thermal vents environments contain negligible amounts of phytoplankton, the occurrence of 18:1($n$-7) or 16:1($n$-7) FA can be directly linked to bacterial production. Hydrothermal vent species like the tube worm *Riftia pachyptilla*, *Mundiopsis subsquamosa*, *Bathymodiolus* sp. or *Bythograea thermydron* contain significant amounts of these bacterial markers (Phleger et al. 2005a; Phleger et al. 2005b). Not only in deep sea, but also shallow water hydrothermal habitats feeding on bacteria was reported to change lipid compositions significantly (Kharlamenko et al. 1995). For example, Kharlamenko et al. (1995) reported, that the symbiont containing clam, *Axinopsida orbiculata* exhibited high amounts of 18:1($n$-7), 16:1($n$-7) and 16:0 fatty acids. In respect to this knowledge the presents of high amounts of 18:1($n$-7) (25% of total FA) and 16:1($n$-7) FA (10% of total FA) in *X. testudinatus* mid gut glands strongly suggests a utilization of bacteria. The question whether these trophic markers have an herbal origin or not can be answered by the fact, that no significant amounts of algae were observed in this habitat. Furthermore, an uptake of these FA by phytoplankton previously ingested with the killed zooplankton would not produce such high concentrations of these FA. The behaviour of grazing and ingesting filamentous bacteria, growing on the body of *X. testudinatus* was observed during scuba diving and in captivity. Regarding these findings one can assume, that besides the feeding on killed zooplankton, these crabs are able to utilize an alternative food source: bacteria.

## 5. Conclusion and prospects

It has been demonstrated that this shallow water hydrothermal vent crab exhibits physiological adaptations towards their specialized feeding behavior in this highly toxic habitat. These physiological properties are mainly reflected by the digestive features and the energy storage. A digestive tract designed for omnivorous feeding combined with a set of highly active enzymes enables this species to efficiently utilize major food compounds, which only occasionally occur. Furthermore, proteolytic enzymes like, Trypsin and Chymotrypsin, can be characterised by high stabilities in terms of thermal degradation, pH stability and inhibition by metal ions. These features can be regarded as essential for an organism to function efficiently over long exposure times, in this hydrothermal habitat. High amounts of lipid reserves in the mid gut gland, in order to overcome prolonged periods of food scarcity, corroborate with the specialized feeding on killed zooplankton only occurring in absence of currents or strong winds. Lipid reserves of this crab are not sufficient to cover energetic demand during 30 days starvation under routine respiratory conditions. These findings indicate a decrease of metabolic rates whose mechanisms remain unanswered. The lipids are mainly stored as TAGs although long time reserves like WE are also present in low amounts. The fatty acid composition underlines the expected carnivorous feeding by high amounts of the FATM 18:1($n$-9). Furthermore, the presence of fatty acids from the MUFA (n-7) series indicates a dietary bacterial input, assured by the fact that except of bacteria high amounts of other MUFA (n-7) sources, like diatoms or algae are lacking in this habitat.

This work solely investigated the feeding physiology of *Xenograpsus* adults and thus questions about physiological properties of larva stages remain unknown. In this context interesting aspects can be outlined as following: Are the larvae lecithotrophic, or do they rely on an external food source? Do digestive features and energy storage characteristics vary with ontogenetic levels? What are the physiological mechanisms to facilitate or avoid dispersal? The role of vent associated bacteria that seem to be utilized by *X. testudinatus* remains unclear for other animals observed in this area and opens up a topic of great interest. Can vent associated bacteria also serve as a food source in shallow water hydrothermal vent systems?

The shallow water hydrothermal vent habitat of Kueishan Island holds active fumaroles and solfataras discharging sulphides and metal ions comparable to those on the mid ocean ridges. Answers to the beforehand mentioned questions are of high interest, and may serve as a suitable model to explain biological processes at deep sea hydrothermal vents.

**This work has demonstrated that:**

- *X. testudinatus* has similar metabolic demands to other tropical benthic decapods
- *X. testudinatus* exhibits highly active digestion enzymes adapted to an irregular food supply
- Digestive enzymes display high stabilities to cope with the toxic habitat
- digestive capacities of this crab correlate with food availability
- *X. testudinatus* is capable to store significant amounts of TAG in the mid gut gland
- this species is able to overcome long starvation periods
- the presence of fatty acid carnivory markers indicates the feeding on zooplankton from a molecular point of view
- Behavioral aspects and FATM from the MUFA (*n*-7) series indicate a supplementary bacteriovorous mode of nutrition in *X. testudinatus*

## 6. Acknowledgement/ Danksagung

I would like to thank/Ich danke:

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## 8. Appendix

*Raw data collected during this work and evaluated in the present thesis*

**Contents**

**Table A1** shows oxygen consumption rates in respect to different temperatures. Outliners are marked with a hash-symbol and were excluded

| O$_2$ Consumption [µmol*g$_{FW}$$^{-1}$*h$^{-1}$] | | | | |
|---|---|---|---|---|
| No. | 15°C | 20°C | 25°C | 30°C |
| 1 | 1.38 | 2.05 | 1.34 | 7.22 |
| 2 | 1.12 | 2.58 | 4.29 | 4.36 |
| 3 | 1.33 | 2.07 | 4.18 | 3.36 |
| 4 | 0.68 | 1.08 | 4.04 | 3.74 |
| 5 | #-0.282 | 2.26 | 2.26 | 3.67 |
| 6 | 1.11 | 2.78 | #1.246 | 4.43 |
| 7 | 1.13 | 3.83 | 4.23 | 7.52 |
| 8 | 0.91 | 2.77 | 3.97 | 3.91 |
| 9 | 0.90 | 2.37 | 3.79 | 6.58 |
| 10 | 0.98 | 2.94 | 2.91 | 5.50 |
| 11 | | | 3.00 | 3.36 |
| 12 | | | 4.54 | 5.19 |
| 13 | | | 3.30 | 4.15 |
| 14 | | | 3.94 | 7.24 |
| 15 | | | #6.101 | 3.33 |

**Table A2** Temperature dependent transit times of *X. testudinatus*

| 15°C | | 20°C | | 25°C | | 30°C | |
|---|---|---|---|---|---|---|---|
| no. | Transit time | no. | Transit time | no. | Transit time | no. | Transit time |
| 1 | 6.25 | 1 | 4.67 | 1 | 5.50 | 1 | 4.63 |
| 2 | 4.25 | 2 | 4.67 | 2 | 5.50 | 2 | 4.13 |
| 3 | 7.25 | 3 | 8.17 | 3 | 3.50 | 3 | 5.13 |
| 4 | 7.25 | 4 | 5.17 | 4 | 5.00 | 4 | 5.63 |
| 5 | 6.75 | 5 | 5.17 | 5 | 4.50 | 5 | 4.63 |
| 6 | 6.25 | 6 | 8.17 | 6 | 4.50 | 6 | 4.63 |
| 7 | 6.25 | 7 | 7.67 | 7 | 6.50 | 7 | 5.63 |
| 8 | 7.25 | 8 | 6.67 | 8 | 6.50 | 8 | 6.63 |
| 9 | 7.25 | 9 | 7.17 | 9 | 6.00 | 9 | 1.63 |
| 10 | 7.25 | 10 | 7.67 | 10 | 6.00 | 10 | 6.13 |

**Table A3** ApiZym enzyme scan of 20 digestive enzymes of *X. testudinatus*

| Enzyme | colour intensity | Enzyme | colour intensity |
|---|---|---|---|
| Ester hydrolases | | Phosphoric hydrolases | |
| Esterase (C 4) | 2 | Alkaline phosphatase | 5 |
| | 2 | | 5 |
| Esterase Lipase (C8) | 3 | Acid phosphatase | 4 |
| | 3 | | 4 |
| Lipase (C 14) | 0 | Naphtol- | 4 |
| | 0 | AS-BI-phosphohydrolase | 4 |
| Glucosidases | | | |
| α-galactosidase | 1 | | |
| | 1 | | |
| β-galactosidase | 4 | Peptide hydrolases | |
| | 4 | | |
| β-glucuronidase | 4 | Leucin arylamidase | 5 |
| | 4 | | 5 |
| α-glucosidase | 4 | Valin arylamidase | 3 |
| | 5 | | 3 |
| β-glucosidase | 3 | Cysteine arylamidase | 2 |
| | 3 | | 2 |
| N-acetyl-β- | 5 | Trypsin | 5 |
| Glucosaminidase | 5 | | 5 |
| α-mannosidase | 3 | α-chymotrypsin | 0 |
| | 3 | | 0 |
| α-fucosidase | 4 | | |
| | 4 | | |

**Table A4** represents temperature dependent activities of Trypsin and Chymotrypsin. Measurements were performed in duplicates for three individualsin. Average of duplicates are given in this table.

| Temperature | 5℃ | 10℃ | 15℃ | 20℃ | 25℃ | 30℃ | 40℃ | 50℃ | 60℃ | 70℃ |
|---|---|---|---|---|---|---|---|---|---|---|
| Trypsin | 0.45 | 0.88 | 1.66 | 2.86 | 4.59 | 7.11 | 14.17 | 21.51 | 28.39 | 0.57 |
| $[U^*g_{FW}^{-1}]$ | 0.26 | 0.38 | 0.60 | 1.10 | 1.99 | 3.30 | 7.96 | 13.90 | 23.62 | 1.52 |
| | 0.82 | 1.03 | 1.92 | 3.69 | 6.34 | 10.55 | 22.08 | 32.83 | 36.62 | 0.87 |
| Chymotrypsin | 7.52 | 13.01 | 21.77 | 29.47 | 39.11 | 55.52 | 94.60 | 149.07 | 162.91 | 42.73 |
| $[U^*g_{FW}^{-1}]$ | 11.03 | 11.03 | 17.97 | 30.96 | 52.28 | 73.71 | 110.74 | 201.51 | 172.95 | 26.34 |
| | 13.80 | 18.41 | 29.37 | 46.56 | 61.27 | 75.63 | 147.80 | 222.81 | 171.82 | 34.68 |

**Table A5** (a), (b), (c) shows residual specific activities [$\Delta E*min^{-1}*g_{FW}^{-1}$] for Trypsin (a), Chymotrypsin (b) and total protease (C) depending on temperature and exposure times. Measurements for Trypsin and Chymotrypsin were performed with pooled samples of four individuals (n=4), determined in repeat determinations. Residual total protease activity was also performed with pooled samples (n=4) in triplicates. Averages of these triplicates are presented in table 5 (c).

(a)

Trypsin

| Time [min] | 0 | 30 | 60 | 120 | 180 | 240 | 300 |
|---|---|---|---|---|---|---|---|
| 0℃ | 0.063 | | | | | | 0.060 |
| | 0.057 | | | | | | 0.062 |
| 20℃ | 0.063 | 0.065 | 0.063 | 0.062 | 0.065 | 0.060 | 0.060 |
| | 0.057 | 0.068 | 0.061 | 0.059 | 0.060 | 0.062 | 0.062 |
| 40℃ | 0.063 | 0.060 | 0.065 | 0.059 | 0.056 | 0.064 | 0.060 |
| | 0.057 | 0.056 | 0.058 | 0.061 | 0.064 | 0.059 | 0.063 |
| 50℃ | 0.063 | 0.066 | 0.072 | 0.069 | 0.075 | 0.063 | 0.064 |
| | 0.057 | 0.061 | 0.065 | 0.065 | 0.066 | 0.067 | 0.061 |
| 55℃ | 0.063 | 0.065 | 0.078 | 0.071 | 0.079 | 0.073 | 0.072 |
| | 0.057 | 0.069 | 0.073 | 0.080 | 0.069 | 0.079 | 0.068 |
| 60℃ | 0.063 | 0.077 | 0.087 | 0.076 | 0.078 | 0.067 | 0.067 |
| | 0.057 | 0.072 | 0.075 | 0.091 | 0.064 | 0.073 | 0.064 |
| 65℃ | 0.063 | 0.044 | 0.044 | 0.035 | 0.032 | 0.024 | 0.017 |
| | 0.057 | 0.047 | 0.041 | 0.036 | 0.029 | 0.025 | 0.017 |
| 70℃ | 0.063 | 0.026 | 0.009 | 0.003 | 0.002 | 0.002 | 0.003 |
| | 0.057 | 0.021 | 0.009 | 0.006 | 0.007 | 0.001 | 0.002 |
| 80℃ | 0.063 | 0.008 | 0.005 | 0.002 | 0.002 | 0.003 | 0.001 |
| | 0.057 | 0.005 | 0.006 | 0.005 | 0.002 | 0.002 | 0.002 |

(b)

Chymotrypsin

| Time [min] | 0 | 30 | 60 | 120 | 180 | 240 | 300 |
|---|---|---|---|---|---|---|---|
| 0℃ | 0.381 | | | | | | 0.387 |
| | 0.379 | | | | | | 0.362 |
| 20℃ | 0.387 | 0.365 | 0.355 | 0.376 | 0.379 | 0.382 | 0.374 |
| | 0.362 | 0.363 | 0.346 | 0.363 | 0.368 | 0.380 | 0.362 |
| 40℃ | 0.387 | 0.373 | 0.388 | 0.386 | 0.380 | 0.405 | 0.401 |
| | 0.362 | 0.379 | 0.372 | 0.375 | 0.394 | 0.397 | 0.413 |
| 50℃ | 0.381 | 0.410 | 0.407 | 0.377 | 0.396 | 0.385 | 0.378 |
| | 0.379 | 0.439 | 0.378 | 0.416 | 0.408 | 0.383 | 0.401 |
| 55℃ | 0.387 | 0.373 | 0.348 | 0.332 | 0.258 | 0.265 | 0.254 |
| | 0.362 | 0.357 | 0.372 | 0.344 | 0.289 | 0.243 | 0.227 |
| 60℃ | 0.381 | 0.227 | 0.141 | 0.063 | 0.027 | 0.006 | 0.002 |
| | 0.379 | 0.222 | 0.155 | 0.066 | 0.027 | 0.007 | 0.002 |
| 70℃ | 0.381 | 0.001 | 0.001 | 0.000 | 0.000 | 0.002 | 0.000 |
| | 0.379 | 0.001 | 0.000 | 0.000 | 0.000 | 0.002 | 0.000 |

(c)

Total protease (Pooled 6,7,8,9)

| Time [min] | 0 | 30 | 60 | 120 | 180 | 240 | 300 |
|---|---|---|---|---|---|---|---|
| 0℃ | 0.373 | | | | | | 0.369 |
| 20℃ | 0.373 | 0.382 | 0.394 | 0.361 | 0.387 | 0.404 | 0.390 |
| 40℃ | 0.373 | 0.392 | 0.399 | 0.402 | 0.423 | 0.432 | 0.433 |
| 50℃ | 0.373 | 0.423 | 0.402 | 0.376 | 0.372 | 0.402 | 0.415 |
| 55℃ | 0.373 | 0.405 | 0.394 | 0.447 | 0.338 | 0.381 | 0.352 |
| 60℃ | 0.373 | 0.320 | 0.280 | 0.254 | 0.136 | 0.138 | 0.122 |
| 70℃ | 0.373 | 0.043 | 0.043 | 0.026 | 0.010 | 0.008 | 0.006 |

**Table A6** shows pH dependent Trypsin and Chymotrypsin activities (Spec. Act. $[\Delta E*min^{-1}*g_{FW}^{-1}]$) measured in duplicates. Additionally Activities are presented as % of the highest value determined for each sample (n=4)

|  | Chymotrypsin | | Trypsin | |
|---|---|---|---|---|
|  | Spec. Act. | In % | Spec. Act. | In % |
| pH2 | 0.000E+00 | 0.00 | 1.323E-05 | 0.43 |
|  | 0.000E+00 | 0.00 | 7.032E-06 | 0.24 |
|  | 1.602E-05 | 0.05 | 4.805E-05 | 1.41 |
|  | 0.000E+00 | 0.00 | 3.406E-05 | 1.53 |
| pH4 | 1.838E-04 | 0.73 | 8.233E-05 | 2.69 |
|  | 1.603E-04 | 0.67 | 5.907E-05 | 1.98 |
|  | 2.611E-04 | 0.77 | 6.566E-05 | 1.93 |
|  | 1.480E-04 | 0.74 | 3.876E-05 | 1.74 |
| pH6 | 9.133E-03 | 36.38 | 1.120E-03 | 36.56 |
|  | 1.060E-02 | 44.14 | 1.160E-03 | 38.82 |
|  | 1.307E-02 | 38.36 | 1.324E-03 | 38.94 |
|  | 8.810E-03 | 44.25 | 8.680E-04 | 38.98 |
| pH8 | 2.511E-02 | 100.00 | 2.642E-03 | 86.23 |
|  | 2.400E-02 | 100.00 | 2.781E-03 | 93.04 |
|  | 3.408E-02 | 100.00 | 3.209E-03 | 94.35 |
|  | 1.991E-02 | 100.00 | 2.032E-03 | 91.24 |
| pH10 | 1.185E-03 | 4.72 | 3.064E-03 | 100.00 |
|  | 1.378E-03 | 5.74 | 2.989E-03 | 100.00 |
|  | 1.731E-03 | 5.08 | 3.402E-03 | 100.00 |
|  | 9.984E-04 | 5.01 | 2.227E-03 | 100.00 |

**Table A7** shows residual activities of Trypsin and Chymotrypsin after incubation at the respecting pH for 30 min (Spec. Act. $[\Delta E*min^{-1}*g_{FW}^{-1}]$). Measured were performed in duplicates. Additionally Activities are presented as % of the highest value determined for each sample (n=4)

|  | Chymotrypsin | | Trypsin | |
|---|---|---|---|---|
|  | Spec. Act. | In % | Spec. Act. | In % |
| pH2 | 0.000E+00 | 0.00 | 1.323E-05 | 0.43 |
|  | 0.000E+00 | 0.00 | 7.032E-06 | 0.24 |
|  | 1.602E-05 | 0.05 | 4.805E-05 | 1.41 |
|  | 0.000E+00 | 0.00 | 3.406E-05 | 1.53 |
| pH4 | 1.838E-04 | 0.73 | 8.233E-05 | 2.69 |
|  | 1.603E-04 | 0.67 | 5.907E-05 | 1.98 |
|  | 2.611E-04 | 0.77 | 6.566E-05 | 1.93 |
|  | 1.480E-04 | 0.74 | 3.876E-05 | 1.74 |
| pH6 | 9.133E-03 | 36.38 | 1.120E-03 | 36.56 |
|  | 1.060E-02 | 44.14 | 1.160E-03 | 38.82 |
|  | 1.307E-02 | 38.36 | 1.324E-03 | 38.94 |
|  | 8.810E-03 | 44.25 | 8.680E-04 | 38.98 |
| pH8 | 2.511E-02 | 100.00 | 2.642E-03 | 86.23 |
|  | 2.400E-02 | 100.00 | 2.781E-03 | 93.04 |
|  | 3.408E-02 | 100.00 | 3.209E-03 | 94.35 |
|  | 1.991E-02 | 100.00 | 2.032E-03 | 91.24 |
| pH10 | 1.185E-03 | 4.72 | 3.064E-03 | 100.00 |
|  | 1.378E-03 | 5.74 | 2.989E-03 | 100.00 |
|  | 1.731E-03 | 5.08 | 3.402E-03 | 100.00 |
|  | 9.984E-04 | 5.01 | 2.227E-03 | 100.00 |

**Table A8** presents Trypsin and Chymotrypsin activities in the presence of inorganic inhibitors and EDTA. Activities [$\Delta E * min^{-1} * g_{FW}^{-1}$] were determined in duplicates for three individuals (n=3).

| | Chymotrypsin | Trypsin | | Chymotrypsin | Trypsin |
|---|---|---|---|---|---|
| Control | 1.76E-02 | 2.17E-03 | MgSO$_4$ | 1.69E-02 | 1.60E-03 |
| | 1.93E-02 | 2.21E-03 | | 2.18E-02 | 1.55E-03 |
| | 2.21E-02 | 2.40E-03 | | 2.57E-02 | 2.65E-03 |
| | 2.16E-02 | 2.41E-03 | | #0.0347 | 2.59E-03 |
| | 1.41E-02 | 2.37E-03 | | 1.74E-02 | 2.60E-03 |
| | 1.73E-02 | 2.29E-03 | | 1.78E-02 | 2.56E-03 |
| | | | | | |
| CuCl$_2$ | 3.23E-03 | 6.78E-04 | HgNO$_3$ | 1.86E-02 | 1.80E-03 |
| | 3.33E-03 | 7.99E-04 | | 1.65E-02 | 1.90E-03 |
| | 5.82E-03 | 7.21E-04 | | 2.77E-02 | 2.23E-03 |
| | 5.51E-03 | 7.62E-04 | | 2.28E-02 | 2.39E-03 |
| | 2.92E-03 | 4.06E-04 | | 1.50E-02 | 2.54E-03 |
| | 2.83E-03 | 3.48E-04 | | 1.34E-02 | 2.31E-03 |
| | | | | | |
| LiCl$_2$ | 1.62E-02 | 1.75E-03 | AlCl$_2$ | 1.06E-02 | 1.15E-03 |
| | 1.74E-02 | 1.98E-03 | | 1.15E-02 | 1.06E-03 |
| | 2.38E-02 | 2.28E-03 | | 1.86E-02 | 1.13E-03 |
| | 2.31E-02 | 2.18E-03 | | 1.87E-02 | 1.14E-03 |
| | 1.76E-02 | 2.22E-03 | | 1.34E-02 | 1.02E-03 |
| | 1.59E-02 | 1.86E-03 | | 1.36E-02 | 8.36E-04 |
| | | | | | |
| CoCl$_2$ | 1.44E-02 | 1.91E-03 | NaCl | 2.15E-02 | 2.39E-03 |
| | 1.42E-02 | 1.89E-03 | | 1.88E-02 | 2.43E-03 |
| | 1.61E-02 | 2.65E-03 | | 2.61E-02 | 2.43E-03 |
| | 1.76E-02 | 1.98E-03 | | 2.63E-02 | 2.41E-03 |
| | 1.14E-02 | 1.39E-03 | | 1.28E-02 | 2.60E-03 |
| | 1.16E-02 | 1.23E-03 | | 1.97E-02 | 2.52E-03 |
| | | | | | |
| EDTA | 1.41E-02 | 1.19E-03 | FeCl$_2$ | 7.68E-03 | 1.11E-03 |
| | 9.70E-03 | 1.44E-03 | | 6.34E-03 | 9.53E-04 |
| | 1.88E-02 | 1.95E-03 | | 9.96E-03 | 1.81E-03 |
| | 1.91E-02 | 1.86E-03 | | 1.25E-02 | 1.63E-03 |
| | 8.81E-03 | 2.01E-03 | | 3.61E-03 | 1.40E-03 |
| | 9.93E-03 | 1.97E-03 | | 3.67E-03 | 1.35E-03 |
| | | | | | |
| | | | NaSO$_4$ | 1.73E-02 | 2.41E-03 |
| | | | | 1.70E-02 | 2.55E-03 |
| | | | | 2.84E-02 | 2.46E-03 |
| | | | | 2.45E-02 | 2.68E-03 |
| | | | | 1.27E-02 | 4.19E-03 |
| | | | | 1.35E-02 | 4.10E-03 |

**Table A9**  Protein concentrations and fractions collected by anion exchange chromatography

| Fractions | Extinktion | Fractions | Extinktion | Fractions | Extinktion |
|-----------|-----------|-----------|-----------|-----------|-----------|
| 1 | 0.2 | 24 | 0.4 | 47 | 0.6 |
| 2 | 0.2 | 25 | 0.4 | 48 | 0.6 |
| 3 | 0.2 | 26 | 0.6 | 49 | 0.7 |
| 4 | 0.2 | 27 | 0.5 | 50 | 0.6 |
| 5 | 0.2 | 28 | 0.6 | 51 | 0.6 |
| 6 | 0.2 | 29 | 0.7 | 52 | 0.7 |
| 7 | 0.2 | 30 | 2 | 53 | 0.8 |
| 8 | 0.2 | 31 | 3.8 | 54 | 1 |
| 9 | 0.2 | 32 | 3.2 | 55 | 0.8 |
| 10 | 0.3 | 33 | 1.8 | 56 | 1 |
| 11 | 0.8 | 34 | 3.2 | 57 | 0.8 |
| 12 | 2.2 | 35 | 2.6 | 58 | 0.6 |
| 13 | 1.7 | 36 | 1.6 | 59 | 1.1 |
| 14 | 1 | 37 | 2.2 | 60 | 0.7 |
| 15 | 2.4 | 38 | 1.3 | 61 | 0.6 |
| 16 | 2 | 39 | 1.1 | 62 | 0.5 |
| 17 | 1.4 | 40 | 1.7 | 63 | 0.4 |
| 18 | 0.6 | 41 | 1.3 | 64 | 0.4 |
| 19 | 0.4 | 42 | 1.5 | 65 | 0.3 |
| 20 | 0.8 | 43 | 0.8 | 66 | 0.2 |
| 21 | 1 | 44 | 0.8 | 67 | 0.1 |
| 22 | 0.8 | 45 | 1 | 68 | 0 |
| 23 | 0.4 | 46 | 0.8 | | |

**Table A10**  Protease and Lipase specific activities [$\Delta E * min^{-1} * g_{FW}^{-1}$]  of fed and starved specimens

| | Starvation (Day 0) | Starvation (Day 10) | Starvation (Day 20) | Starvation (Day 30) |
|---|---|---|---|---|
| protease | 0.34 | 0.42 | 0.25 | 0.36 |
| | #0.24 | 0.40 | 0.34 | 0.23 |
| | 0.63 | #0.63 | 0.19 | 0.38 |
| | 0.47 | 0.42 | 0.36 | 0.29 |
| | 0.51 | 0.41 | 0.36 | 0.21 |
| | 0.50 | 0.36 | 0.47 | 0.39 |
| | 0.39 | 0.46 | #0.68 | 0.30 |
| | | | | |
| lipase | 3.40 | 1.23 | 1.03 | #2.85 |
| | 2.56 | 1.23 | 0.59 | 1.23 |
| | 2.46 | 0.78 | 0.71 | 0.93 |
| | 1.72 | 0.42 | 0.37 | 0.59 |

**Table A11** shows lipid contents of Fed (1-10), starved 10 days (11-20), starved 20 days (21-30) and starved 30 days (31-39) individuals. Including hepatopancreas dry mass (DM), and measured lipid in order to calculate specific amounts. Outliners are marked with a hash-symbol and were excluded.

| No. | DM | Lipid [mg] | Specific amount [%] | mg Lipid/mg DM |
|-----|--------|-----------|---------------------|----------------|
| 1   | 122.23 | 46.75     | #38.21              | 0.38           |
| 2   | 238.08 | 123.52    | 51.86               | 0.52           |
| 3   | 228.27 | 108.29    | 47.42               | 0.47           |
| 4   | 113.39 | 63.80     | 56.23               | 0.56           |
| 5   | 113.63 | 55.90     | 49.15               | 0.49           |
| 6   | 131.51 | 67.85     | 51.67               | 0.52           |
| 7   | 153.33 | 79.54     | 51.93               | 0.52           |
| 8   | 246.41 | 148.58    | 60.34               | 0.60           |
| 9   | 184.83 | 107.20    | 58.05               | 0.58           |
| 10  | 202.34 | 110.43    | 54.62               | 0.55           |
| 11  | 24.76  | 1.43      | #7.1                | 0.07           |
| 12  | 145.29 | 60.39     | 41.80               | 0.42           |
| 13  | 108.95 | 41.51     | 38.40               | 0.38           |
| 14  | 58.06  | 11.98     | 21.21               | 0.21           |
| 15  | 144.99 | 66.29     | 45.95               | 0.46           |
| 16  | 222.71 | 142.69    | 64.22               | 0.64           |
| 17  | 137.27 | 71.44     | 52.28               | 0.52           |
| 18  | 107.14 | 49.48     | 46.50               | 0.46           |
| 19  | 109.99 | 42.07     | 38.56               | 0.39           |
| 20  | 76.15  | 35.05     | 46.46               | 0.46           |
| 21  | 137.18 | 71.43     | 52.12               | 0.52           |
| 22  | 92.66  | 36.07     | 39.02               | 0.39           |
| 23  | 175.30 | 103.79    | 59.25               | 0.59           |
| 24  | 131.46 | 67.19     | 51.17               | 0.51           |
| 25  | 80.14  | 28.31     | 35.43               | 0.35           |
| 26  | 148.65 | 70.55     | 47.51               | 0.48           |
| 27  | 135.39 | 64.92     | 48.01               | 0.48           |
| 28  | 42.11  | 7.85      | 18.84               | 0.19           |
| 29  | 93.42  | 36.14     | 38.78               | 0.39           |
| 30  | 114.94 | 42.44     | 37.00               | 0.37           |
| 31  | 163.48 | 95.38     | 58.32               | 0.58           |
| 32  | 79.91  | 29.08     | 36.33               | 0.36           |
| 33  | 202.68 | 81.27     | 40.07               | 0.40           |
| 34  | 141.87 | 71.42     | 50.31               | 0.50           |
| 35  | 68.70  | 21.69     | 31.50               | 0.31           |
| 36  | 61.72  | 15.33     | 24.99               | 0.25           |
| 37  | 57.35  | 10.17     | 17.89               | 0.18           |
| 38  | 102.93 | 59.28     | 57.68               | 0.58           |
| 39  | 49.71  | 7.05      | 14.38               | 0.14           |

**Table A12** Mid gut lipid classes of *X. testudinatus* given in % of TL

| Starvation (Day 0) | | | | | | Starvation (Day 30) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample No. | WE/SE | TAG | FFA | ST | PL | Sample No. | WE/SE | TAG | FFA | ST | PL |
| 1a | 4.84 | 82.19 | 1.36 | 0.88 | 10.73 | 31a | 1.00 | 97.37 | 0.14 | 0.27 | 1.22 |
| 1b | 2.81 | 85.72 | 1.73 | 0.23 | 8.27 | 31b | 0.97 | 96.33 | 0.08 | 0.29 | 2.33 |
| 2a | 4.52 | 90.30 | 1.50 | 0.68 | 3.00 | 32a | 1.15 | 88.86 | 0.98 | 0.01 | 9.00 |
| 2b | 4.12 | 92.42 | 0.11 | 0.75 | 2.59 | 32b | 2.16 | 83.70 | 1.68 | 2.88 | 9.58 |
| 3a | 3.88 | 91.14 | 0.32 | 0.85 | 3.81 | 33a | 4.62 | 90.70 | 0.33 | 0.74 | 3.62 |
| 3b | 3.70 | 90.90 | 0.08 | 0.89 | 4.42 | 33b | 4.20 | 92.51 | 0.21 | 0.00 | 3.08 |
| 4a | 1.24 | 95.91 | 0.11 | 0.50 | 2.24 | 34a | 2.85 | 91.97 | 0.44 | 0.92 | 3.82 |
| 4b | 3.80 | 93.39 | 0.37 | 0.37 | 2.07 | 34b | 2.67 | 93.27 | 0.06 | 0.79 | 3.21 |
| 5a | 0.75 | 96.64 | 0.53 | 0.70 | 1.37 | 35a | 1.91 | 81.82 | 0.46 | 1.51 | 14.29 |
| 5b | 0.47 | 93.94 | 0.07 | 0.54 | 4.98 | 35b | 1.31 | 79.51 | 1.60 | 2.19 | 15.39 |
| 6a | 1.58 | 90.57 | 0.04 | 1.05 | 6.76 | 36a | 0.50 | 70.42 | 0.63 | 4.43 | 24.02 |
| 6b | 2.25 | 92.30 | 0.26 | 0.85 | 4.34 | 36b | 7.41 | 62.91 | 0.68 | 4.08 | 24.92 |
| 7a | 2.16 | 91.43 | 1.01 | 2.01 | 3.38 | 37a | 2.15 | 51.17 | 2.14 | 12.37 | 32.17 |
| 7b | 1.81 | 94.16 | 0.47 | 0.28 | 3.28 | 37b | 1.92 | 46.41 | 1.65 | 8.53 | 41.49 |
| 8a | 1.63 | 95.76 | 0.19 | 0.52 | 1.91 | 38a | - | - | - | - | - |
| 8b | 1.41 | 95.90 | 0.19 | 0.41 | 2.09 | 38b | 1.46 | 93.65 | 0.22 | 1.11 | 3.56 |
| 9a | 2.06 | 87.38 | 5.56 | 1.12 | 3.88 | 39a | 0.69 | 28.03 | 2.88 | 11.23 | 57.18 |
| 9b | 1.92 | 92.56 | 0.23 | 0.88 | 4.40 | 39b | 7.93 | 30.54 | - | 0.28 | 61.25 |
| 10a | 2.28 | 93.88 | 0.55 | 0.50 | 2.79 | | | | | | |
| 10b | 1.72 | 94.28 | 0.19 | 1.06 | 2.76 | | | | | | |

**Table A13** Mid gut gland fatty acids of *X. testudinatus* given in % of TL

| Fatty acids | Starvation (day 0) Mean ± S.D. (10) | Starvation (day 10) Mean ± S.D. (10) | Starvation (day 20) Mean ± S.D. (10) | Starvation (day 30) Mean ± S.D. (9) |
|---|---|---|---|---|
| 14:0 | 1.1 ± 0.3 | 2.0 ± 0.7 | 2.3 ± 0.4 | 0.7 ± 0.3 |
| 14:1(*n*-5) | 0.1 ± 0.1 | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.0 ± 0.1 |
| 15:0 | 0.3 ± 0.1 | 0.4 ± 0.2 | 0.5 ± 0.1 | 0.3 ± 0.2 |
| 14:0A | 0.0 ± 0.1 | 0.3 ± 0.2 | 0.3 ± 0.1 | 0.1 ± 0.1 |
| 16:0 | 16.5 ± 5.8 | 18.8 ± 3.3 | 20.4 ± 0.8 | 18.0 ± 3.2 |
| 16:1(*n*-7) | 10.8 ± 1.2 | 11.1 ± 1.8 | 11.4 ± 1.9 | 8.3 ± 1.7 |
| 16:1(*n*-5) | 0.5 ± 0.1 | 0.7 ± 0.2 | 0.6 ± 0.1 | 0.5 ± 0.1 |
| 16:2(*n*-4) | 0.6 ± 0.2 | 0.5 ± 0.2 | 0.5 ± 0.2 | 0.6 ± 0.1 |
| 17:0 | 0.5 ± 0.1 | 0.5 ± 0.1 | 0.5 ± 0.1 | 0.6 ± 0.2 |
| 16:3(*n*-4) | 0.4 ± 0.1 | 0.4 ± 0.2 | 0.4 ± 0.1 | 0.4 ± 0.2 |
| 16:0A | 0.1 ± 0.1 | 0.3 ± 0.2 | 0.4 ± 0.2 | 0.3 ± 0.1 |
| 18:0 | 5.2 ± 0.6 | 5.1 ± 1.0 | 5.0 ± 0.6 | 5.8 ± 0.7 |
| 18:1(*n*-9) | 11.3 ± 1.7 | 13.0 ± 3.0 | 11.4 ± 2.3 | 11.8 ± 0.9 |
| 18:1(*n*-7) | 22.8 ± 2.2 | 18.9 ± 6.4 | 21.7 ± 3.4 | 24.6 ± 4.7 |
| 18:1(*n*-5) | 0.4 ± 0.1 | 0.4 ± 0.1 | 0.4 ± 0.0 | 0.5 ± 0.2 |
| 18:2(*n*-4) | 0.4 ± 0.1 | 0.3 ± 0.1 | 0.4 ± 0.0 | 0.3 ± 0.1 |
| 18:2(*n*-6) | 1.1 ± 0.3 | 1.4 ± 0.7 | 1.0 ± 0.4 | 1.1 ± 0.4 |
| 18:3(*n*-6) | 0.3 ± 0.1 | 0.1 ± 0.2 | 0.1 ± 0.1 | 0.3 ± 0.1 |
| 18:3(*n*-3) | 0.6 ± 0.1 | 0.7 ± 0.2 | 0.6 ± 0.2 | 0.5 ± 0.2 |
| 18:4(*n*-3) | 0.5 ± 0.3 | 0.2 ± 0.3 | 0.0 ± 0.0 | 0.2 ± 0.2 |
| 20:0 | 0.4 ± 0.1 | 0.5 ± 0.2 | 0.0 ± 0.0 | 0.5 ± 0.1 |
| 20:1(*n*-11) | 0.4 ± 0.0 | 0.4 ± 0.1 | 0.2 ± 0.2 | 0.4 ± 0.2 |
| 20:1(*n*-9) | 0.9 ± 0.2 | 0.9 ± 0.4 | 0.7 ± 0.5 | 1.0 ± 0.2 |
| 20:1(*n*-7) | 2.1 ± 0.4 | 1.9 ± 0.8 | 2.1 ± 0.4 | 2.7 ± 0.9 |
| 20:2(*n*-6) | 0.4 ± 0.3 | 0.3 ± 0.2 | 0.3 ± 0.1 | 0.4 ± 0.2 |
| 20:3(*n*-6) | 0.2 ± 0.1 | 0.2 ± 0.2 | 0.1 ± 0.2 | 0.3 ± 0.1 |
| 20:4(*n*-6) | 2.0 ± 0.5 | 2.4 ± 1.7 | 1.9 ± 0.8 | 2.6 ± 1.1 |
| 20:4(*n*-3) | 0.4 ± 0.1 | 0.4 ± 0.2 | 0.3 ± 0.2 | 0.2 ± 0.1 |
| 20:5(*n*-3) | 4.9 ± 1.1 | 4.7 ± 1.8 | 4.2 ± 1.0 | 4.7 ± 1.5 |
| 22:0 | 0.4 ± 0.1 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.5 ± 0.1 |
| 22:1(*n*-11) | 0.5 ± 0.1 | 0.6 ± 0.4 | 0.5 ± 0.2 | 0.5 ± 0.2 |
| 22:5(*n*-3) | 0.8 ± 0.2 | 0.7 ± 0.3 | 0.6 ± 0.2 | 0.6 ± 0.3 |
| 22:6(*n*-3) | 7.7 ± 1.7 | 6.4 ± 2.6 | 5.9 ± 1.7 | 6.3 ± 2.2 |
| unknown | 4.1 ± 2.5 | 4.7 ± 3.4 | 3.3 ± 0.8 | 3.6 ± 1.4 |
| | | | | |
| Sum SAT | 24.4 ± 7.0 | 27.3 ± 5.4 | 28.7 ± 2.0 | 26.4 ± 4.8 |
| Sum MUFA | 49.9 ± 6.2 | 48.1 ± 13.4 | 49.3 ± 9.3 | 50.3 ± 9.1 |
| Sum PUFA | 20.5 ± 5.3 | 18.4 ± 8.9 | 16.4 ± 5.3 | 18.4 ± 6.6 |
| Sum (*n*-7) | 35.7 ± 3.8 | 32.0 ± 8.9 | 35.3 ± 5.7 | 35.6 ± 7.3 |
| 18:1(n-7)/18:1(n-9) | 2.0 | 1.5 | 1.9 | 2.1 |

Fatty acid composition (mass% of total fatty acids) of starved (0, 10, 20, 30 days) specimens presented as mean and standard deviation (S.D.); SAT (saturated fatty acids), MUFA (monounsaturated fatty acids), PUFA (polyunsaturated fatty acids) and (*n*-7) (fatty acids from the (*n*-7) series); (n) Number of specimens tested.

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**Eidesstattliche Erklärung**

</div>

Hiermit erkläre ich, dass die vorliegende Arbeit von mir selbstständig durchgeführt und ausgearbeitet worden ist. Es wurde lediglich auf die angegebene Literatur bezug genommen. Ich versichere weiterhin, dass diese Arbeit noch keinem anderen Prüfungsgremium vorgelegen hat.

Bremen, den 21.12. 2007

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Marian Yong-An Hu