Metabolic energy demand and food utilization of the hydrothermal vent crab *Xenograpsus testudinatus* (Crustacea: Brachyura)

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ABSTRACT: The hydrothermal vent crab *Xenograpsus testudinatus* (Crustacea: Brachyura) is endemic near Kueishan Island, Taiwan, where it lives in shallow waters close to the hydrothermal vents located in this area. *X. testudinatus* is adapted to a sulfur-rich and thus potentially toxic environment. It has established a specialized feeding strategy focusing on dead zooplankton organisms killed by the toxic discharges from the vents. During slack water, when there is little or no current, the crabs leave their crevices to feed on this 'marine snow'. In the present study, we investigated the physiological aspects of nutritional adaptations of *X. testudinatus*. The crabs showed high digestive capacities of major digestive enzymes and particularly high activities for proteolytic enzymes. This feature can be regarded as an adaptation to irregular food availability. Furthermore, enzymes were stable at elevated temperatures, in a wide pH range, and in the presence of inorganic inhibitors like Cu^{2+}, Fe^{2+}, or Co^{2+}. These enzyme properties can be considered essential to functioning in a vent habitat over long exposure times. Moreover, *X. testudinatus* is able to store significant amounts of lipid (50 to 60% of dry mass in the midgut gland), which may help to overcome periods of food scarcity. Fatty acid profiles revealed high amounts of saturated and monounsaturated components (mainly 16:0, 16:1(n-7), 18:1(n-9), and 18:1(n-7)). These findings reflect physiological adaptations and energetic strategies that enable this crab to exist in this extreme hydrothermal vent habitat.

KEY WORDS: Hydrothermal vents · Crustacea · *Xenograpsus testudinatus* · Digestive enzymes · Fatty acids · Toxic environment · Heavy metals

INTRODUCTION

Five volcanic islands rise up along the axis of the Okinawa Trough back-arc basin, which extends from the northeast of Taiwan to the Unzen volcano in Kyushu. The southwesternmost island is Kueishan Dao ('Turtle Mountain Island'; 24° 25′ N, 121° 57′ E). The hydrothermal vents are located at depths of 8 to 20 m and are characterized by low pH (1.8 to 4.6) and sulfur-rich discharges which reach temperatures of 65 to 116°C. Moreover, the vents release various gases, mainly carbon dioxide, nitrogen, oxygen, sulfur dioxide, and hydrogen sulfide (Jeng et al. 2004).

This unique environment is inhabited by the hydrothermal vent crab *Xenograpsus testudinatus*, which is endemic to this area. It lives close to the hydrother-

mal vents and is one of the few known vent-endemic species at shallow depths of <200 m. *X. testudinatus* is the only species in the immediate surroundings of the hydrothermal vents. In the vicinity (50 m), *X. testudinatus* still dominate, but 3 other benthic species, including the snail *Nassarius* sp., the hexacoral *Tubastraea aurea*, and a sessile polychaete (Serpulidae), plus one species of algae (Corallinaceae) and the fish *Siganus fuscescens* have also been recorded (Chen et al. 2005b).

The waters near the hydrothermal vents are poor in nutrients but rich in trace elements such as Fe, Cu, Al, and Mn (Chen et al. 2005a). *Xenograpsus testudinatus* has an opportunistic and specialized feeding behavior, mainly preying upon dead zooplankton killed by the toxic discharges from the vents. The preference for this food has been indicated by gut content analysis that showed mainly pelagic copepods in crab stomachs (Jeng et al. 2004). During slack water, when there is little or no current, the crabs leave their crevices and feed rapidly on this 'marine snow' of dead planktonic organisms (Jeng et al. 2004). The crabs are thus exposed to toxins in 2 ways: (1) they are exposed to the toxins dissolved in the seawater, and (2) they ingest toxins with the prey, which was previously killed by the toxins in the water. Moreover, the crabs are able to cope with short exposures to low pH and high water temperatures.

Due to the discontinuous and poor food supply, the ability of *Xenograpsus testudinatus* to store significant amounts of lipids can be regarded as crucial in surviving prolonged periods of food depletion. It would allow the animal to feed when prey abundance is high and utilize endogenous lipid stores during periods of food paucity.

Fatty acids (FA) derived from triacylglycerols (TAG) and other storage lipids may serve as trophic biomarkers (Dalsgaard et al. 2003). The FA 18:1 (n-9) can provide information about carnivory, based on the fact that this FA is a major component in most marine animals and accumulates in food chains (Dalsgaard et al. 2003). FA profiles are available for deep-sea hydrothermal vent species that rely on a bacteria-based food source (Pond et al. 1998, Phleger et al. 2005a,b). These species show high amounts of vaccenic (18:1(n-7)) acid, which is used as trophic marker for sulfur-oxidizing bacteria (SOX) in hydrothermal vent environments (Pond et al. 2002, Dalsgaard et al. 2003, Phleger et al. 2005a). High concentrations of palmitoleic (16:1(n-7)) and vaccenic (18:1(n-7)) acids, on the other hand, are not unambiguous indicators for SOX because 16:1(n-7) is also abundant in many algae, usually diatoms, making it

difficult to discriminate between a chemosynthetic or photosynthetic contribution of FA (Van Dover 2000), and 18:1(n-7) is also a general indicator of anaerobic bacterial input (Tunlid & White 1992).

The anatomy of the digestive system and biochemical digestive features have provided major clues in understanding feeding and nutrition in hydrothermal vent species (Van Dover et al. 1988, Boetius & Felbeck 1995). The digestive systems of many sessile or sluggish deep-sea hydrothermal vent species like tubeworms, clams, or mussels are degenerated but contain 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 bacteriophagous feeding (González et al. 1993). In contrast to these organisms, which can be considered primary consumers, there are predatory species like the crab *Bythograea thermydron*, which feeds on vestimentiferans and mussels (Phleger et al. 2005b). This crab shows low lysozyme activity, but instead, higher activities of proteolytic and lipolytic enzymes (Boetius & Felbeck 1995).

The present work is aimed at extending our knowledge about the nutritional adaptations of *Xenograpsus testudinatus* from a physiological point of view. It comprises the determination of the energetic demands of the species by measuring routine oxygen consumption as well as the identification of the catalytic properties of important digestive enzymes. Furthermore, aspects of energy storage and trophic preferences were addressed by investigating the midgut gland lipid levels, lipid class and FA compositions. The overall goal of the present work is to help elucidate how these crabs can successfully inhabit an environment otherwise hostile to life and whether they show specific ecophysiological or biochemical adaptations.

MATERIALS AND METHODS

Sampling of crabs

Experiments with living specimens of *Xenograpsus testudinatus* were carried out in the laboratories of the Institute for Biodiversity, Academia Sinica (Taiwan) during summer 2007. Two hundred male specimens with carapace widths between 2 and 3 cm were collected during SCUBA-diving at the hydrothermal venting area (depth: 8 to 15 m) near Kueishan Island (Fig. 1) and transported in aerated cooling boxes to the laboratory. Only adult males were sampled in
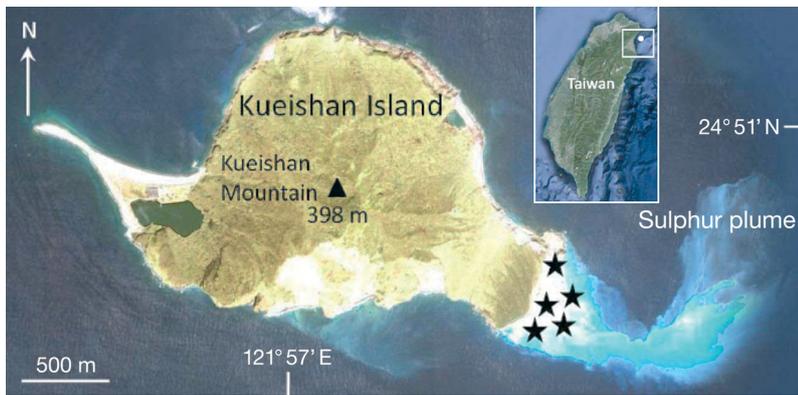
Fig. 1. Kueishan Island located off Taiwan's east coast. Hydrothermal vent area is delimited by stars. Source: Google Earth, photographed May 16, 2004, and accessed October 20, 2011

order to minimize artefacts in lipid and enzyme analysis due to sexual maturation and vitellogenesis. The withdrawal of the 200 specimens from their environment did not affect the population, which was estimated to comprise some 3.6 million crabs (supplement in Jeng et al. 2004). In the laboratory, the crabs were maintained in 250 l tanks filled with natural seawater (salinity: 33 to 35) and connected with an external pump (Alife, AE-1060) to a bottom gravel filter system. The crabs were kept under a 12 h light: 12 h dark regime at 24°C.

Crabs used for starvation experiments were maintained in separate plastic jars (500 ml) to avoid aggression or cannibalism. The jars were punctured to ensure water exchange. Eight to 9 of these jars were placed in 60 l tanks equipped with external filters (Alife, AE-1060). At the start of the experiments (Day 0), 20 specimens, which were caught the same day, were anesthetized by cooling on ice and then killed by an incision in the frontal region of the carapace. The midgut gland from each specimen was excised, weighed, immediately frozen in liquid nitrogen, and thereafter stored at −80°C. Another 80 specimens were maintained individually in the jars. Every 10 d, 20 specimens were treated and sampled as described for Day 0. At each sampling date, 10 midgut glands were used for lipid analysis and the remaining 10 for enzyme assays. There was no crab mortality during the 30 d experiment.

Foregut morphology

The stomachs of 8 specimens of *Xenograpsus testudinatus* were dissected on the same day the animals were captured. Sections through all 3 planes were made, and samples were treated according to a scanning electron microscopy (SEM) protocol (Lee et al. 1996), including prefixation in 4% paraformaldehyde with 5% glutaraldehyde (P4G5) for 10 h. Subsequently, samples were transferred into a 0.1 mol l^{-1} phosphate buffer (PB) and washed 3 times. In order to maintain cellular structures, membrane fixation was performed with 1% OsO$_4$ in 0.1 mol l^{-1} PB for 30 min under a hood. After fixation, samples were again washed in 0.1 mol l^{-1} PB. The samples were dehydrated in 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 examined in a SEM (FEI Quanta 200) within an electrical field of 25 kV.

Respiration measurements

Prior to the determination of routine metabolic rates, crabs were acclimated for 1 wk to the experimental conditions. Oxygen consumption rates were determined in 5.8 l respiration chambers at atmospheric pressure. Five chambers were filled with filtered seawater (0.2 µm) and equipped with 1 crab each. A sixth chamber without a crab served as a control. For acclimation, starved animals (24 h) were pre-incubated in the respiration chambers for 3 h, while the water was aerated to reach full oxygen saturation. Depending on the experimental temperature (15, 20, 25 or 30°C), crabs were incubated for 5 to 12 h in the dark. In order to keep the incubation temperature constant, test chambers were placed in a water bath equipped with a Dixell Prime temperature control device that was connected to the circulation incubator (Dixell, Superflite). A decrease of oxygen concentrations below 70% was avoided. The appropriate incubation time for each temperature was empirically determined in preliminary experiments. Before and after incubation, water samples (3 × 50 ml plus overflow) were carefully siphoned off the respiration chamber, and the amount of dissolved oxygen was determined following the Winkler method (Hansen 1999). For each respiration measurement, 5 new crabs were used.

In order to determine oxygen consumption of bacteria derived from the surfaces of the crabs, the

remaining seawater in each chamber was again tested for oxygen consumption, but without crabs. The water was aerated in order to saturate it with oxygen. The chambers were again incubated for 5 h and the oxygen concentrations were measured as described in the previous paragraph. The amount of oxygen consumed by bacteria was subtracted from the overall oxygen consumption of the crabs.

The relationship between oxygen consumption of the crabs and the water temperature was demonstrated by calculating Q_{10} values according to van 't Hoff's rule for metabolic processes:

$$Q_{10} = \frac{R_2}{R_1}^{10K(T_2 - T_1)} \tag{1}$$

where R_1 and R_2 represent the respiration rates at temperatures T_1 and T_2.

Lipid analysis

Frozen midgut glands were lyophilized for 48 h and the dry mass was determined. Lipid extraction was done following Hagen (2000). The lyophilized samples were mechanically homogenized (Sartorius; Potter S) and extracted in dichloromethane (DCM)/methanol (2:1 v/v). The amount of total extractable lipid was determined gravimetrically on a microbalance (Sartorius precision balance R200D, precision: 10 µg) and presented as % of dry mass (%DM).

Lipid extracts adjusted to a final concentration of 14 mg ml^{-1} in DCM were used to determine lipid class composition using thin layer chromatography with flame ionization detection (Iatron Laboratories, MK-5) according to Fraser et al. (1985).

FA analysis

Subsamples of lipid extracts (100 µg) were used for trans-esterification. After evaporation of the solvent under a continuous flow of nitrogen, 1 ml of methanolic sulfuric acid (3%) and 250 µl of hexane were added to the sample and the mixture was incubated under nitrogen for 4 h at 80°C. Fifty µl of sample were adjusted to a concentration of 0.02 mg ml^{-1} hexane and were then analyzed by gas chromatography (GC) according to Kattner & Fricke (1986). The GC (HP 6890) was equipped with a cold-injection system and a DB-FFAP column (30 m, inner diameter of 0.25 mm, 0.25 µm coating). Fatty acid methyl esters (FAMEs) were quantified by a flame ionization

detector and identified by comparison with retention times of an established fish oil standard (Marinol).

Preparation of crude enzyme extract

Samples (50 to 100 mg) of deep-frozen midgut glands of *Xenograpsus testudinatus* were extracted in 1 ml of demineralized water. The tissues were homogenized with an ultrasonic cell disruptor (Branson Sonifier, B15) with 30% of maximum energy and 3 bursts of 3 s each with a break of 7 s in between. The homogenates were centrifuged for 15 min at 15 000 g and 4°C. The supernatants were then transferred into new reaction cups and used for further enzyme analysis. All steps were performed on ice in order to avoid thermal degradation or enzymatic proteolysis. For the characterization of proteases, we used only samples from freshly caught animals that were deep-frozen within 6 h after capture.

Protein concentrations of the crude extracts were determined according to Bradford (1976) with a commercial protein kit (Bio-Rad) and bovine serum albumin as standard.

Api Zym enzyme assays

The Api Zym system (bioMérieux, REF 25200) was used following Donachie et al. (1995) and Saborowski et al. (2006) to screen *Xenograpsus testudinatus* midgut gland extracts for a set of 19 digestive enzymes (see Table 3). Fifty µl of the midgut gland crude extracts (50 mg ml^{-1}) were added to each well on the test trays. The trays were incubated at 30°C for 2 h. Then 1 drop of each of the reagents Zym A and Zym B were added to the wells. The enzyme activities were classified semi-quantitatively according to the intensities of the developed colors. Assays were run in duplicate. However, this assay is not sensitive for crustacean chymotrypsin activity, due to the inappropriate substrate *N*-glutaryl-phenylalanine-2-naphthylamide.

Protease activity and stability

The activities of the serine proteases trypsin and chymotrypsin were determined colorimetrically with the substrates *N*-benzoyl-L-arginine-4-nitroanilide-hydrochloride (BAPNA; Fluka 12915) for trypsin and *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (SAAPNA; Sigma S7388) for chymotrypsin. In brief, 20 µl of

crude extract were added to 960 µl of 0.1 mol l^{-1} Tris·HCl-buffer (pH 7) and pre-incubated for 3 min. The reaction was started by addition of 20 µl of substrate (50 mmol l^{-1} in DMSO). The increase of absorbance at 405 nm was measured in a spectrophotometer at constant temperatures from 5 to 70°C.

The thermal stability of the enzymes was examined in pooled extracts from 4 individuals. Sub-samples were pre-incubated for up to 5 h at 0 to 70°C. Thereafter, samples were cooled on ice and the residual proteolytic activity was subsequently measured as described in the previous paragraph.

The effect of inorganic substances on the activities of proteolytic enzymes was examined with the following metal ions and reagents: $CuCl_2$, $LiCl_2$, $CoCl_2$, NaCl, $FeCl_2$, $AlCl_3$, $MgSO_4$, $HgNO_3$, and EDTA. The concentration of all salts and reagents in the reaction mixtures was 0.01 mol l^{-1}.

The pH profiles of proteases were investigated using universal buffer following Ellis (1961). The pH stability was studied while crude extracts were incubated at pH 2, 4, 6, 8, 10, and 12 for 30 min at 30°C. Subsequently, samples were examined for protease activity at pH 7.

Statistical analysis

Statistical analysis was performed with the computer program SigmaStat 3.1 (Systat). One-way ANOVA, including test of normality, equality of variances, and all pair-wise multiple comparison procedures (Holm-Sidak method) were applied to compare proteolytic activities under varying experimental conditions and during the starvation period. Student's *t*-test was performed to evaluate differences among lipid profiles during starvation.

RESULTS

On June 3, 2007 we collected *Xenograpsus testudinatus* and, simultaneously, had the unique opportunity to observe this species in its natural habitat. The crabs were sampled in 8 to 15 m depth. The habitat was poor in flora and fauna and smelled of sulfurous plumes discharged by the vents. During SCUBA-diving we observed how a group of crabs fed on a dead flying fish, which had probably been killed by the toxic plumes. This observation shows that *X. testudinatus* also scavenges on other available food sources besides zooplankton.

Foregut morphology

The nomenclature of labeled components (Figs. 2 & 3) and the terminology of the description of the foregut essentially follows Martin et al. (1998). The median tooth of *Xenograpsus testudinatus* can be separated in 2 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 2 groups of striking projecting setae at the posterior edge of the tooth (Figs. 2 & 3a). The urocardiac ossicle is smooth and resembles a tongue carrying the median tooth on its posterior end. The lateral teeth are well developed
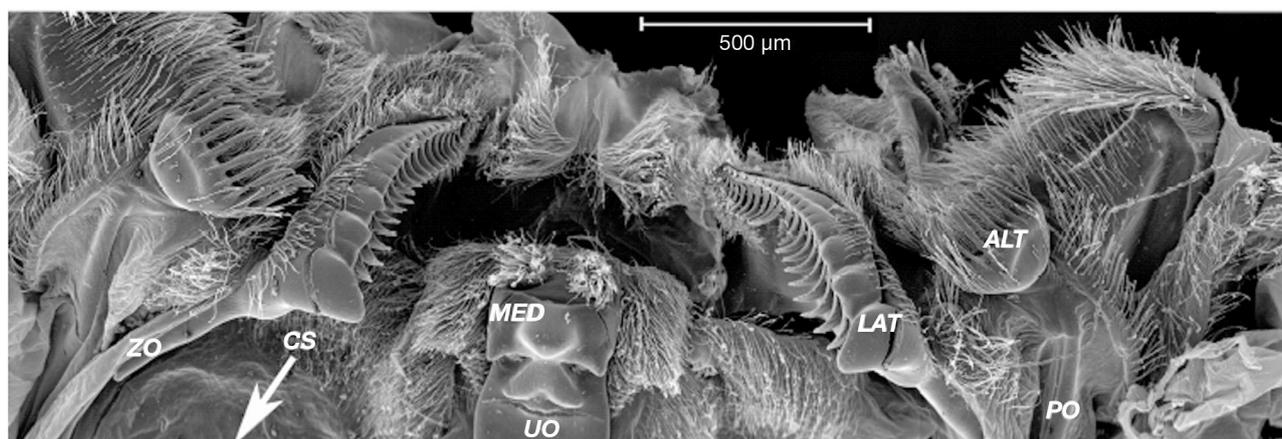
Fig. 2. *Xenograpsus testudinatus*. Scanning electron microscopy (SEM) image of internal anatomy of the stomach. Dorsal view on the open stomach revealing the median tooth (MED), lateral tooth (LAT), accessory lateral tooth (ALT), urocardiac ossicle (UO), zygocardiac ossicle (ZO), and prepectineal ossicle (PO). The position of the cardiac stomach (CS; not shown) is indicated by the arrow
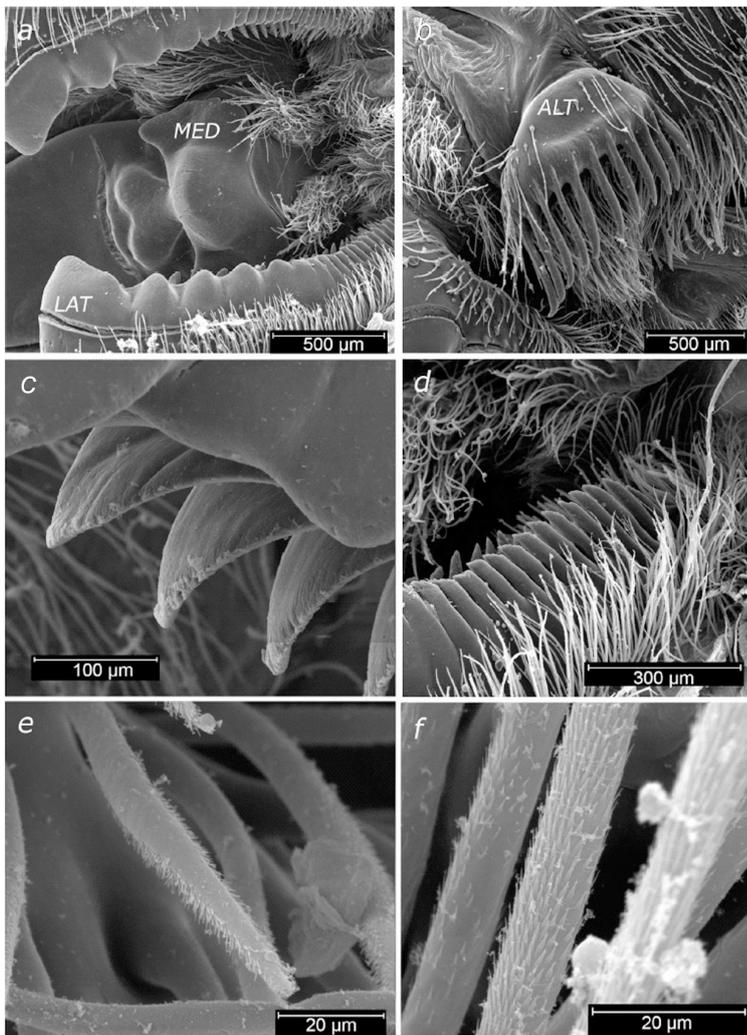
Fig. 3. *Xenograpsus testudinatus*. Scanning electron microscopy (SEM) photographs of foregut ossicles and setae covering the internal surface of the foregut. (a) Lateral teeth (LAT) and median tooth (MED); note the setae covering the dorsal surface of the lateral tooth. (b) Close-up of accessory lateral teeth (ALT) from anterior view, showing the 11 medially directed spines. Detailed image of the blade-shaped lateral teeth from (c) lateral and (d) dorsal view. (e,f) Fine structure of the setae, showing 2 types of setae, differing in their denticle distribution

and carry one large tooth at their anterior end. The posterior part consists of about 18 blade-shaped teeth (Figs. 2 & 3c), which become smaller in the 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 stomach of *X. testudinatus*, bear denticles (Fig. 3e,f). Accessory teeth are paired, attached to prepectineal ossicles, and located dorsally in the anterior part of the lateral teeth (Fig. 3b). These accessory ossicles bear 11 medially directed spines, which carry denticles at their end. Only a 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 2 types of setae. The first type consists of a central, slightly flattened body carrying 2 rows of laterally 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. 3e). The second type of setae is cylindrical, carrying small denticles that cover one side of the round setae (Fig. 3f). The second type is widely distributed throughout the whole foregut.

Oxygen consumption

The routine oxygen consumption (ROC) of *Xenograpsus testudinatus* increased with temperature from 1.1 ± 0.2 µmol g_{FM}^{-1} h^{-1} (where FM is fresh mass; error values are SD throughout this paper) at 15°C to 4.9 ± 1.5 µmol g_{FM}^{-1} h^{-1} at 30°C. At 25°C it was 3.5 ± 1.0 µmol g_{FM}^{-1} h^{-1} (Fig. 4). The Q_{10} value calculated over the whole temperature range was 3.13. Q_{10} values for the temperature ranges 20 to 25°C and 25 to 30°C were approximately 2. Between 15 and 20°C, the Q_{10} value was 5.43.

Respiration data allowed us to calculate the metabolic energetic demand. Assuming that protein is a major food source of *Xenograpsus testudinatus*, we used a respiratory quotient of 0.85 (Nelson et al. 1977). At an ambient temperature of 25°C, average-sized adult *X. testudinatus* specimens of 10 g_{FM} release 27.4 µmol CO_2 g_{FM}^{-1} h^{-1}. This is equal to 0.0789 g C 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). Therefore, the daily energy demand of a specimen with 10 g body mass amounts to 0.525 kJ d^{-1}, or 525 ± 71 J d^{-1} (= 0.125 ± 0.017 kcal d^{-1}).

Lipid content and composition

Mean total lipid contents of the midgut glands of *Xenograpsus testudinatus* ranged from 53.5 ± 4.2 %DM in freshly sampled animals to 36.8 ± 16.3 %DM in ani-
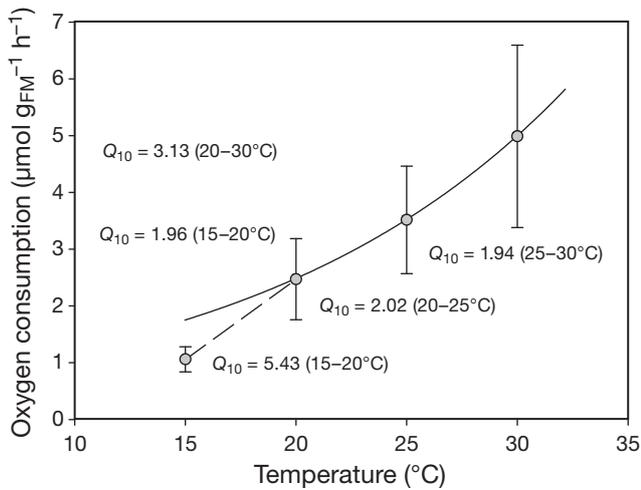
Fig. 4. *Xenograpsus testudinatus*. Temperature-dependent oxygen consumption. Between 20 and 30°C, an exponential regression was applied in accordance with van 't Hoff's rule. Oxygen consumption rates at 20 and 15°C are connected by a dashed line, indicating a deviation from the exponential course. Q_{10} values are given for each temperature interval tested and additionally for a theoretical value between 15 and 20°C (dashed). Error bars indicate SD (n = 9 to 14). FM: fresh mass

mals starved for 30 d (Table 1). Due to high variability, the differences were not statistically significant.

The majority of the lipids in the midgut gland were TAG (neutral lipids) and polar lipids, which together accounted for 95% of total lipids (%TL) in fed and in starved animals (Table 2). Free fatty acids as well as sterols contributed only marginally to total lipids. After starvation for 30 d, the amount of TAG declined from 92.0 ± 2.5 %TL to 76.3 ± 23.5 %TL. Simultaneously, the proportion of polar lipids increased from 4.0 ± 2.2 %TL to 17.4 ± 19.6 %TL. These results show a decrease of TAG during starvation, but due to higher variation among starved animals, the differences between fed and starved specimens were not statistically significant.

Table 1. *Xenograpsus testudinatus*. Lipid contents of midgut glands on Day 0 and after starvation for 30 d. Values are given in % of midgut gland dry mass (DM). Lipid classes are presented as % of total lipids (TL) of 10 individual crabs analyzed. Values are mean ± SD

	Day 0	Starvation for 30 d
Total lipids (%DM)	53.5 ± 4.2	36.8 ± 16.3
Lipid classes (%TL)		
Wax/sterol esters	2.4 ± 1.2	2.6 ± 1.3
Triacylglycerols	92.0 ± 3.5	76.3 ± 23.5
Free fatty acids	0.7 ± 0.9	1.0 ± 0.9
Sterols	0.8 ± 0.2	2.9 ± 3.4
Polar lipids	4.0 ± 2.2	17.4 ± 19.6

FA composition

The FA compositions of fed and starved specimens were dominated by saturated (SFA) and monounsaturated fatty acids (MUFA), while only a few polyunsaturated fatty acids (PUFA) were present (Table 2). The major FA was the MUFA 18:1(n-7), which accounted for 22.8 ± 2.3 % (field samples) and 24.7 ± 4.6 % (starved 30 d) of total FA. Other MUFAs were 16:1(n-7) (~10% of total FA) and 18:1(n-9) (~12% of total FA). The SFA 16:0 amounted to ~17% and 18:0 to about 5% of total FA. The major PUFAs, 22:5(n-3) and 22:6(n-3) together, only contributed 11% to total FA. During the 30 d of starvation, no statistically significant changes in FA patterns were evident. Only 16:1(n-7) decreased significantly after 30 d of starvation.

Api Zym enzyme assays

The semi-quantitative enzyme screening of midgut gland extracts of *Xenograpsus testudinatus* revealed a wide range of enzyme activities (Table 3). Almost

Table 2. *Xenograpsus testudinatus*. Fatty acid (FA) composition (mass% of total FA) of total lipid extracts of midgut glands. SFA: saturated FA, MUFA: monounsaturated FA, PUFA: polyunsaturated FA, and (n-7): FAs from the (n-7) series. Fatty alcohols (<4%) were excluded. Values are mean ± SD of 10 animals

Fatty acid	Duration of starvation (d)			
	0	10	20	30
14:0	1.1 ± 0.3	2.0 ± 0.7	2.3 ± 0.4	0.7 ± 0.3
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
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
18:2(n-6)	1.1 ± 0.3	1.4 ± 0.7	1.0 ± 0.4	1.1 ± 0.4
18:3(n-3)	0.6 ± 0.1	0.7 ± 0.2	0.6 ± 0.2	0.5 ± 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
20:4(n-6)	2.0 ± 0.5	2.4 ± 1.7	1.9 ± 0.8	2.6 ± 1.1
20:5(n-3)	4.9 ± 1.1	4.7 ± 1.8	4.2 ± 1.0	4.7 ± 1.5
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
ΣSFA	23.4 ± 6.8	26.4 ± 5.0	28.2 ± 1.9	25.1 ± 4.4
ΣMUFA	48.9 ± 6.0	47.2 ± 13.0	48.5 ± 8.9	49.4 ± 8.7
ΣPUFA	17.8 ± 4.2	16.6 ± 7.5	14.8 ± 4.6	16.3 ± 5.7
Σ(n-7)	35.7 ± 3.8	32.0 ± 8.9	35.3 ± 5.7	35.6 ± 7.3

Table 3. *Xenograpsus testudinatus*. Enzyme activities determined with the semi-quantitative Api Zym system for freshly caught specimens. Activity was determined visually by strength of coloration and ranked from 0 for no reaction to the maximum intensity of 5. Chymotrypsin tested negative due to an unsuitable substrate (*N*-glutaryl-phenylalanine-2-naphthylamide) for brachyuran chymotrypsin

Enzyme	Color intensity
Ester hydrolases	
Esterase (C4)	2
Esterase lipase (C8)	3
Lipase (C14)	0
Glycosidases	
α-Galactosidase	1
β-Galactosidase	4
β-Glucuronidase	4
α-Glucosidase	4–5
β-Glucosidase	3
N-acetyl-β-D-glucosaminidase	5
α-Mannosidase	3
α-Fucosidase	4
Phosphoric hydrolases	
Alkaline phosphatase	5
Acid phosphatase	4
Naphthol-AS-BI-phosphohydrolase	4
Peptide hydrolases	
Leucine arylamidase	5
Valine arylamidase	3
Cysteine arylamidase	2
Trypsin	5
α-Chymotrypsin	0

all enzymes tested showed positive reactions, except α-chymotrypsin and lipase (C-14). The highest activity levels, on a scale of 0 to 5 (see Table 3), were shown by leucine arylamidase, trypsin, α-glucosidase, and alkaline phosphatase. Phosphate hydrolases, including alkaline phosphatase, acid phosphatase, and naphthol-AS-BI-phosphohydrolase, showed high activities as well. All glucosidases tested showed intermediate activities.

Effect of starvation on lipolytic and proteolytic enzyme activities

Starvation for 30 d caused a significant decrease in proteolytic as well as lipolytic activities (Fig. 5). At the beginning of the starvation period, the reduction in total proteolytic activity was low but became significant after 20 and 30 d (60 to 70% residual activity). The course of lipase/esterase activity during starvation was similar but the decrease in activity was much more distinct. After 10 d of starvation, the residual lipolytic activity decreased to <60% and after 30 d to about 30% of initial activity.
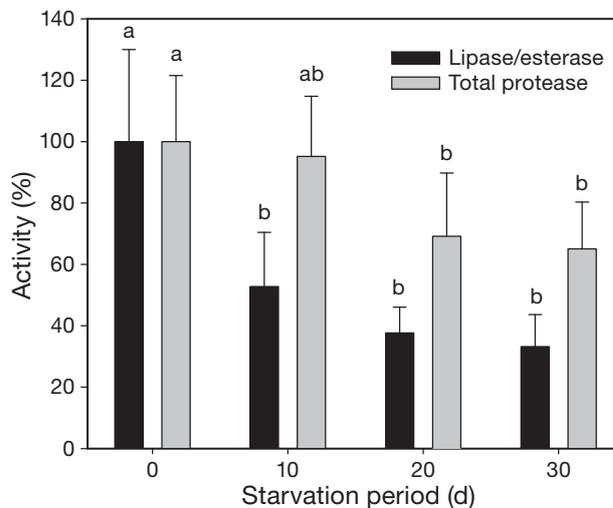
Fig. 5. *Xenograpsus testudinatus*. Changes in the relative activity of lipase/esterase and total protease activity determined photometrically in fed and starved animals (n = 7; mean ± SD). Different letters indicate significant changes in activity within each enzyme group during starvation (Holm-Sidak, p < 0.05)

Effect of temperature on trypsin and chymotrypsin

Enzyme activities measured at the ambient temperature of 25°C were 4.3 ± 2.2 U g^{-1}_{FM} for trypsin and 50.9 ± 11.2 U g^{-1}_{FM} for chymotrypsin. The effects of temperature on midgut gland proteases were investigated with respect to both activity and stability at a given temperature. The temperature/activity curve showed an exponential increase of activity from 5 to 50°C for both trypsin and chymotrypsin (Fig. 6). Chymotrypsin showed maximum activity at 50°C and trypsin at 60°C. At 70°C, however, both enzymes lost almost their entire activities.

The thermal stability assays showed no loss of activity for trypsin and chymotrypsin up to 50°C and to a maximum exposure time of 5 h (Fig. 7). Moreover, trypsin showed increased activities when incubated at temperatures between 40 and 60°C. Significant thermal degradation of trypsin started at 65°C. After 5 h of exposure, the activity dropped to 30% of initial values (Fig. 7a). Chymotrypsin was stable up to 50°C. At 5 h of incubation at 55°C, chymotrypsin started to denature, losing about 40% of initial activity (Fig. 7b).

Effect of pH on trypsin and chymotrypsin

The effects of different pH levels on the stabilities of trypsin and chymotrypsin were investigated using universal buffer adjusted to pH values from 2 to 12 (Fig. 8). The pH stabilities of trypsin and chymo-
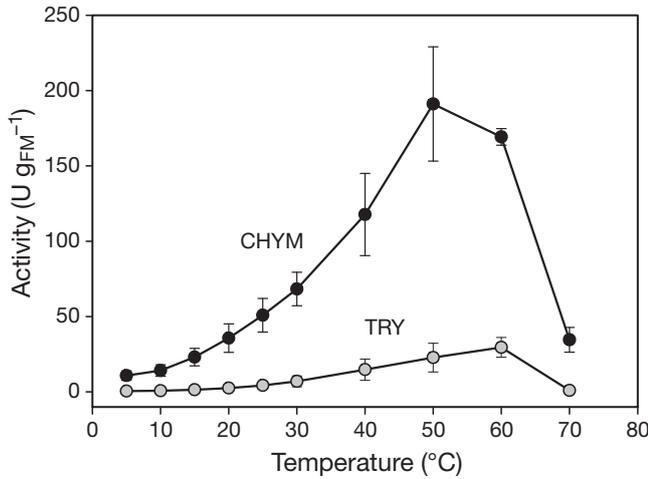
Fig. 6. *Xenograpsus testudinatus*. Temperature-dependent activity curves for the proteases trypsin (TRY) and chymotrypsin (CHYM) (mean ± SD; n = 3). FM: fresh mass
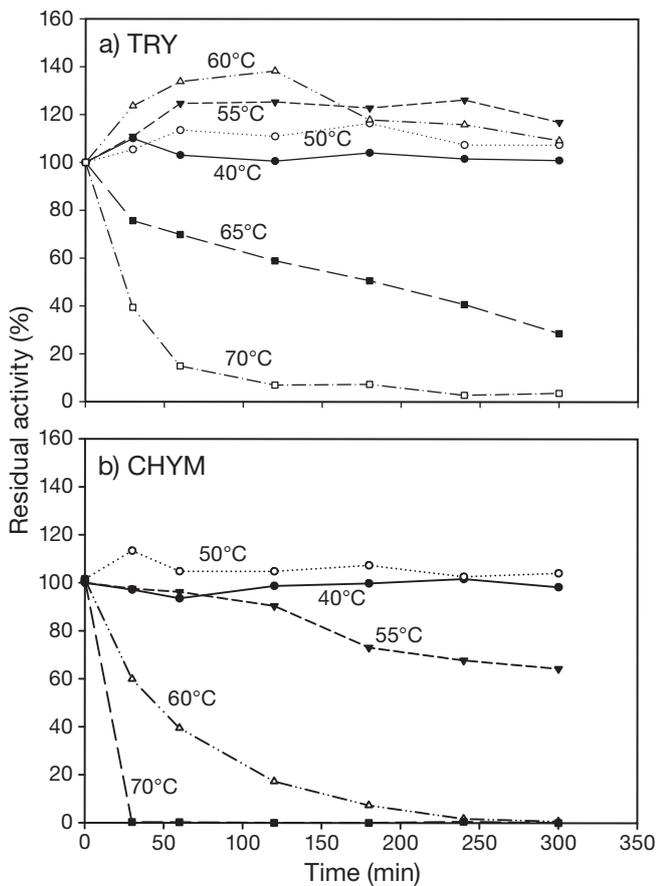
Fig. 7. *Xenograpsus testudinatus*. Thermal stability of the proteases (a) trypsin (TRY) and (b) chymotrypsin (CHYM). Samples from pooled extracts (n = 4 different extracts of 4 individuals each) were incubated at temperatures ranging from 0 to 80°C and exposed to the respective temperature for 0 to 5 h. Residual activity is given as % of the activities measured from samples kept on ice

trypsin showed similar profiles. After 30 min of incubation at pH 2, the residual activities were <10% of maximum activity, 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 the maximum (Fig. 8).

Effects of metal ions on trypsin and chymotrypsin

Metal ions and EDTA had different effects on trypsin and chymotrypsin activities (Fig. 9). The assays with $LiCl_2$, NaCl, $NaSO_4$, $MgSO_4$, and $HgNO_3$ showed no decrease in activities, whereas $CuCl_2$, $CoCl_2$, $AlCl_3$, $FeCl_2$, and EDTA reduced proteolytic activities. Inhibition by copper ions was strongest, leading to residual activities of 20 to 30%. Iron reduced activities by 40 to 60%. Cobalt and EDTA showed low inhibition of 20%. $AlCl_3$ had a different effect on trypsin than on chymotrypsin: trypsin was reduced to 50% of remaining activity, whereas chymotrypsin activity decreased to 80%. Additionally, the effects of sulfurous compounds such as sulfide, sulfate, and sulfite were tested, but these compounds had no significant effects on enzyme activities.

DISCUSSION

The ability of the hydrothermal vent crab *Xenograpsus testudinatus* to survive in an adverse and food-deprived environment mainly relies on feeding
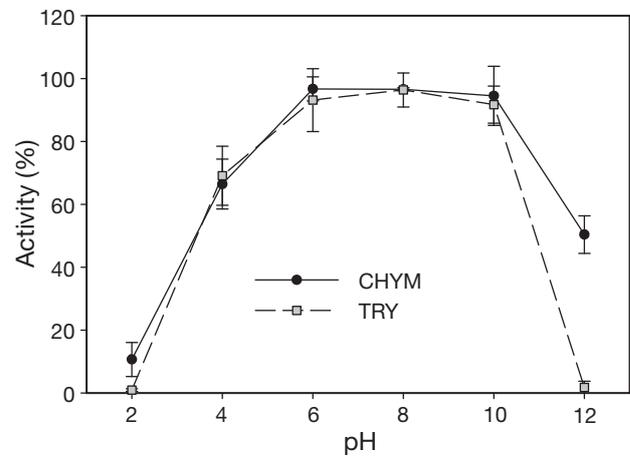
Fig. 8. *Xenograpsus testudinatus*. Effects of pH on protease stability investigated using universal buffer. pH activity measured as residual activity of trypsin (TRY) and chymotrypsin (CHYM) are presented in relation to maximum activity (n = 4 different extracts of 4 individuals each)
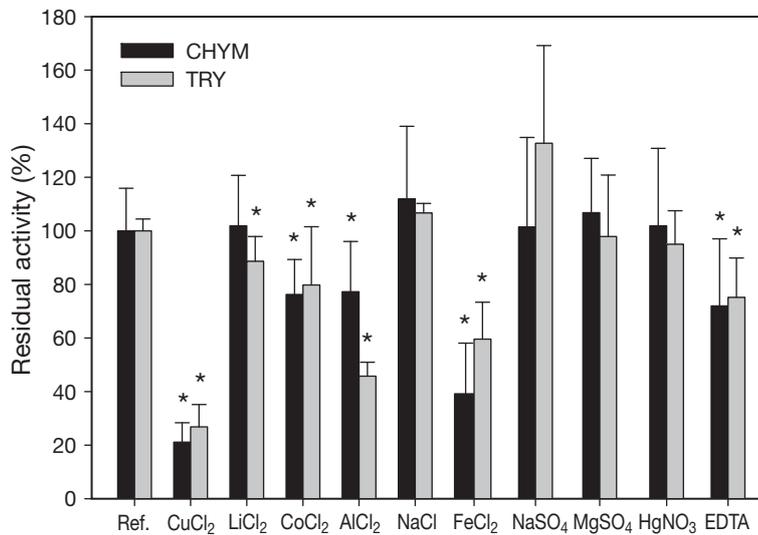
Fig. 9. *Xenograpsus testudinatus*. Effects of inorganic inhibitors on trypsin (TRY) and chymotrypsin (CHYM) activity. Residual activity is presented as % of uninhibited activities (mean ± SD, n = 3). *Significant difference from reference (Ref.) group (Holm-Sidak, p < 0.05)

on dead zooplankton during short periods of slack water (Jeng et al. 2004). The species has developed behavioral, anatomical, and physiological adaptations to cope with the specific conditions around shallow-water hydrothermal vents.

Foregut morphology

Comparison of the foregut morphology of *Xenograpsus testudinatus* with other brachyuran crabs (Meiss & Norman 1977), including the deep-sea hydrothermal vent crab *Bythograea thermydron* (Martin et al. 1998), confirmed that the general orientation of the median tooth, the lateral teeth, and the accessory lateral teeth is similar to that in other brachyurans. The calcified median tooth and lateral teeth are mobile in order to grind food into fine particles. The setae of the foregut are thought to be involved in mixing and filtering the digestive fluids inside the stomach and directing the flow of the chyme to the midgut gland, where the absorption of nutrients takes place (Ceccaldi 1989, Salindeho & Johnston 2003). However, in contrast to the foregut of deep-sea vent crabs described by Martin et al. (1998), the foregut of *X. testudinatus* is densely covered with scales bearing setae in the micrometer range. This high density of setae can be considered advantageous for the specialized feeding of *X. testudinatus* crabs in terms of optimized processing of

ingested food and effective transport of the chyme. The morphology of the gastric mill indicates that *X. testudinatus* is omnivorous (Jeng et al. 2004). Opportunistic feeding, including scavenging, can be considered the most efficient feeding mode for this species.

Energy demand

The increase in respiration of *Xenograpsus testudinatus* with rising temperatures is common for poikilothermic organisms living under atmospheric pressure (Ali et al. 2000, Taylor & Peck 2004, Kunzmann et al. 2007). The magnitude of increase followed van 't Hoff's rule in the temperature range between 20 and 30°C, with Q_{10} values around 2. The deviation of Q_{10} values below 20°C may have resulted from adaptation to higher temperatures and physiological intolerance of low temperatures. Metabolic rates of organisms from polar or temperate regions, like northern krill *Meganyctiphanes norvegica*, follow van 't Hoff's rule in the lower temperature range (Saborowski et al. 2000, 2002, Chausson et al. 2004). In tropical species, van 't Hoff's rule applies to the higher temperature range (Démeusy 1957, Kunzmann et al. 2007). Surface water temperatures in the area of Kueishan Island are usually higher than 20°C (M. S. Jeng pers. obs.), and the temperature at the bottom varies between 20 and 27°C (Chen et al. 2005a). Thus, *X. testudinatus* does not experience water temperatures of ≤15°C in that setting, but in this temperature range in the present study, the crabs showed an excessive reduction in respiration rates.

At 25°C, an individual *Xenograpsus testudinatus* of 10 g body mass converts an energy equivalent of 525 J d^{-1} or 125 cal d^{-1} for maintaining routine metabolic activity. Assuming that 1 g of copepods (ashfree dry mass) contains between ~22 and 31 kJ, a daily zooplankton (copepods) uptake of 85 to 120 mg$_{FM}$ (assuming a mean water content of 80%) is necessary to maintain basic energy demands (Salonen et al. 1976). Taylor & Peck (2004) determined daily energy demands of the sand shrimp *Crangon septemspinosa*. Large shrimps (1.5 g$_{FM}$) metabolize 300 to 400 J d^{-1} at an ambient temperature of 20°C. On a 'per gram' basis, *X. testudinatus* converts 52.5 J and the shrimp 200 to 267 J. This difference is certainly due to the allometric relationship between

body mass and energy demand. But the lower value for *X. testudinatus* may also be explained by reduced activity and a higher amount of calcified shell compared to the shrimp (Weymouth et al. 1944). The shell contributes significantly to the mass of the crab but is otherwise not metabolically active.

Lipid stores

In decapods, the midgut gland is the major lipid-processing and lipid-storage organ (O'Connor & Gilbert 1968, Lawrence 1976, Wen et al. 2006). The ability to accumulate energy stores is an essential physiological prerequisite for survival in environments with deprived or variable food availability. High lipid contents have been reported for sub-polar and polar species, which have to cope with long seasonal starvation periods and tightly coordinated reproduction periods (Albers et al. 1996, Falk-Petersen et al. 2000, Kreibich et al. 2010). However, crabs from temperate or tropical regions may also show elevated lipid contents. The robber crab *Birgus latro* can accumulate lipid contents of up to 75% dry mass (Chakravarti & Eisler 1961), which enables it to survive for >1 yr without food (Storch et al. 1982, Greenaway 2003). *Xenograpsus testudinatus*' midgut gland contained >50% lipids (%DM). Neutral lipids were predominantly stored as TAG. The ability of *X. testudinatus* to store high amounts of lipids can be regarded as beneficial in the animal's habitat. The only input of particulate organic matter (POM) consists of plankton (and nekton) that were killed by toxic discharges and sunk to the ground. This food source is not continuously available and the supply strongly depends on tidal and local currents. Furthermore, Jeng et al. (2004) observed that *X. testudinatus* swarm out and leave their crevices only during slack water. Apparently, the crabs roam and search for food in certain conditions, but they do not seem to actively hunt for food, even when the supply is limited. Accordingly, *X. testudinatus* must be capable of surviving food-deprived periods by using their lipid stores.

Starvation experiments confirmed the ability of *Xenograpsus testudinatus* to survive starvation periods of at least 30 d. The daily energy demands of a 10 g individual *X. testudinatus* amounted to 525 ± 71 J. In turn, the average lipid content in the midgut gland accounted for 75 mg. According to the physical energetic equivalents for lipids (39.7 kJ g^{-1}) (Salonen et al. 1976), the lipid reserves in the midgut gland would theoretically be exhausted after 5 to 6 d. The fact that *X. testudinatus* survived at least 30 d without food indicates that they may catabolize additional energy stores such as proteins or carbohydrates. In addition, *X. testudinatus* may switch to energy-saving metabolic pathways and reduction of metabolic rates. Unfortunately, it was not possible in the frame of the present work to continue respiration measurements after 30 d of starvation, because the availability of crabs was limited. An additional transfer and exposure of crabs from the 30 d starvation experiment to different temperatures could have caused negative effects on the quality of tissue samples that we used for lipid and enzyme analysis.

FA composition

The FA composition of the midgut of *Xenograpsus testudinatus* was dominated by MUFAs of the (n-7) and (n-9) series and the SFA 16:0, but ω3 PUFAs like 20:5(n-3) (eicosapentaenoic acid or EPA) and 22:6(n-3) (docosahexaenoic acid or DHA) were also present. SFA can be synthesized by most species. The MUFA 18:1(n-9) is the major FA in most marine animals and, thus, it may serve as a marker for carnivory (Dalsgaard et al. 2003). Accordingly, high amounts of 18:1(n-9) in the midgut glands of *X. testudinatus* suggest a mainly carnivorous nutrition (Jeng et al. 2004). Moreover, this predominantly carnivorous feeding mode in *X. testudinatus* is supported by low amounts of longer-chain PUFAs such as 20:5(n-3), which are typical markers of diatoms (Kattner & Hagen 1995). Nevertheless, the essential PUFAs of the (n-3) series were present in the midgut gland of *X. testudinatus*. These FAs are predominantly synthesized by primary producers because they possess the enzymes Δ12 and Δ15 desaturase to form (n-3) PUFAs. These essential FAs can directly derive from phytoplankton that co-agglomerates with zooplankton in the marine snow. They may also come from herbivorous or omnivorous zooplankton in which these FAs accumulated.

Many deep-sea vent organisms depend on bacterial primary production. Consequently, all trophic levels are more or less interspersed with bacterial FA trophic markers (Pond et al. 1998, Dalsgaard et al. 2003, Phleger et al. 2005a, 2005b). FAs belonging to the MUFA (n-7) series are predominantly produced by phytoplankton and bacteria, but not by animals (Dalsgaard et al. 2003). Since deep-sea thermal vent environments contain negligible amounts of phytoplankton, 18:1(n-7) or 16:1(n-7) FAs can be directly traced back to bacterial production. Hydrothermal vent species like the tube worm *Riftia pachyptila*, *Munidopsis subsquamosa*, *Bathymodiolus* sp., and

Bythograea thermydron contain significant amounts of these bacterial markers (Phleger et al. 2005a, 2005b). These may also be present in shallow-water hydrothermal habitats. Kharlamenko et al. (1995) showed that the symbiont-containing clam *Axinopsida orbiculata* exhibits high amounts of 18:1(n-7), 16:1(n-7), and 16:0 FAs. However, the origin of 18:1 (n-7) (25% of total FA) and 16:1(n-7) FAs (10% of total FA) in *Xenograpsus testudinatus* midgut glands cannot be clearly traced back to bacteria, as the vents are located within the euphotic zone. It remains to be investigated whether the high concentrations of (n-7) MUFAs, which were a striking feature of the FA profile of *X. testudinatus*, are of bacterial origin or derived from other sources.

Digestive potential

The midgut glands of *Xenograpsus testudinatus* showed a wide range of highly active digestive enzymes comprising various glucanases, esterases, lipases, and peptidases. Since *X. testudinatus* feeds on dead zooplankton, we paid special attention to the important proteolytic enzymes with tryptic and chymotryptic activities (Saborowski et al. 2004, Muhlia-Almazán et al. 2008). The activities of both enzymes were higher than in many other crustacean species measured under the same conditions. The tryptic activities in the midgut gland of freshly caught *X. testudinatus* reached the same levels as in Antarctic krill *Euphausia superba* (Fig. 10), which is known for very high enzyme activities (Anheller et al. 1989,
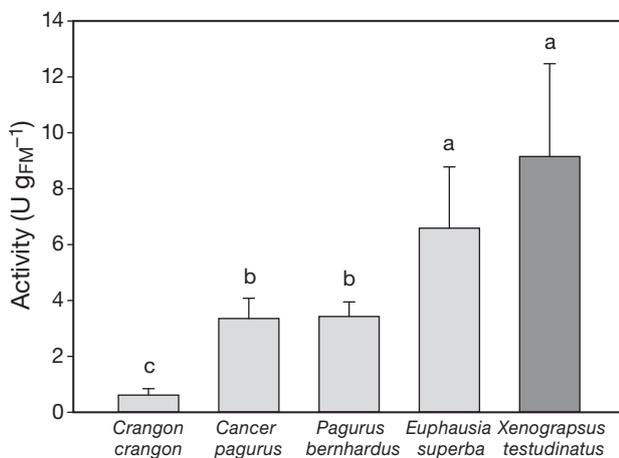
Fig. 10. Comparison of tryptic activity at 30°C in midgut glands of *X. testudinatus* (this study) and several other marine crustaceans (modified after Teschke & Saborowski 2005). Different letters indicate significant differences between groups (Holm-Sidak, p < 0.05). tMean + SD, n = 3 to 6

Turkiewicz et al. 1991). The high digestive capacity of krill has been explained as an adaptation of feeding on patchy and unpredictable phytoplankton (Morris et al. 1983, Saborowski & Buchholz 1999). The animals have to ingest and to utilize their food as fast and efficiently as possible. Similar to Antarctic krill, *X. testudinatus* feeds on an irregular food source, which only appears during slack water. During this short period, the crabs swarm out of their crevices and start to feed rapidly, making the most of the scarce food.

The midgut gland extracts showed high chitinolytic activities of *N*-acetyl-β-glucosaminidase (chitobiase). Crustaceans require chitinolytic enzymes for molting (Oosterhuis et al. 2000) but they also synthesize chitinolytic enzymes in the digestive organs (Saborowski et al. 1993, Peters et al. 1999). High activities of digestive chytinolytic enzymes may indicate that the vent crabs are capable of digesting and utilizing chitin from crustacean shells as a source of both carbon and nitrogen (Saborowski & Buchholz 1999).

Due to high ambient water temperatures, high metal ion concentrations, and extreme pH variations in hydrothermal vent habitats (McMullin et al. 2000), adaptations at the protein/enzyme level appear probable. The trypsin-like proteinase of this species showed remarkable thermal stability and remained unimpaired after exposures to 60°C for 5 h. Compared to other crustaceans (Dittrich 1992, Garcia-Carreño & Haard 1993, Johnston & Freeman 2005), *Xenograpsus testudinatus* proteinases show a significantly elevated thermal tolerance. Dittrich (1992) studied trypsin-like proteases of crustaceans from tropical, temperate, and subarctic regions. The lowest thermal stabilities were present in polar species like the copepod *Calanoides acutus* (loss of >50%, 2 h at 40°C) or the shrimp *Chorismus antarcticus* (loss of >90%, 2 h at 40°C). In contrast, tropical species like the hermit crab *Clibanarius striolatus* and *Ocypode ryderi* retained maximum activities after 2 h at 40°C or 50°C. At 60°C, residual activities in both tropical species totally vanished after 30 and 90 min, respectively (Dittrich 1992). In *X. testudinatus*, the presence of trypsin-like enzymes, which function at elevated temperatures of 60 to 65°C, and proteinases, which function at up to 55°C for 5 h without loss of activity, may be regarded as adaptations to a habitat with temporarily elevated temperatures.

The pH optima of trypsin and chymotrypsin have been investigated in several species (Garcia-Carreño & Haard 1993, Hernández-Cortés et al. 1997, Navarrete del Toro et al. 2006), revealing slightly alkaline working optima. 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 to 8 were observed for trypsins from *Paralithodes camchatica* (Rudenskaya et al. 2000). The present study demonstrates that both trypsin and chymotrypsin of *Xenograpsus testudinatus* show a wide range of stability from slightly acidic to alkaline (pH 6 to 10) conditions. Since the hydrothermal vent site of Kueishan Island is characterized by highly acidic discharges, enzymatic tolerance over a wide pH range can be considered an essential feature of this crab. The hasty and nonselective feeding of *X. testudinatus* during the short slack-water period (Jeng et al. 2004) prohibits a proper selection of food particles. As a consequence, digestive enzymes might be shortly exposed to ingested food particles or water with extreme pH. In order to tolerate these pH variations, a wide pH-stability range can be regarded as an important feature of the crab's digestive enzymes.

The water around the hydrothermal vents contains high concentrations of metal ions such as Mg^{2+}, Ca^{2+}, Fe^{2+}, Cu^{2+}, Al^{3+}, and Mn^{2+} (Chen et al. 2005a). Some of these ions show inhibitory effects on enzymes. Thus, the resistance of digestive enzymes against these inhibitors is an important issue (Dreyfus & Iglewski 1986, Edgcomb et al. 2004, Cardigos et al. 2005). Our results confirm earlier findings that demonstrated that Fe^{2+} and Cu^{2+} inhibit enzyme activities (Sakharov & Prieto 2000). The concentrations reported for hydrothermal vent habitats were usually in the micro- to nanomolar range. Accordingly, the concentrations applied in the present study (10 mmol l^{-1}) can be considered as extremely high for aquatic organisms (Olaifa et al. 2004, Cardigos et al. 2005). Nevertheless, the activities of trypsin and chymotrypsin were not completely inhibited. This indicates a high tolerance against metal ions, which is an advantage for the functioning of these enzymes in the vicinity of hydrothermal vents.

CONCLUSIONS

The shallow-water hydrothermal vent crab *Xenograpsus testudinatus* exhibits physiological adaptations to successfully inhabit a highly challenging environment. These physiological properties are mainly reflected in digestive features and energy storage. A digestive tract designed for omnivorous feeding combined with a set of highly active and stable enzymes enable *X. testudinatus* to efficiently utilize dead zooplankton, which is only available during slack water and absence of currents or strong winds. These features can be regarded as essential for *X. testudinatus* to survive long periods close to the hydrothermal vent system. Substantial lipid reserves in the midgut gland ensure survival during prolonged periods of food scarcity. Future research is needed to study the physiological properties of female and juvenile crabs as well. Also, questions about the distribution and migration of larvae and the ontogenesis of their physiological characteristics should be addressed.

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