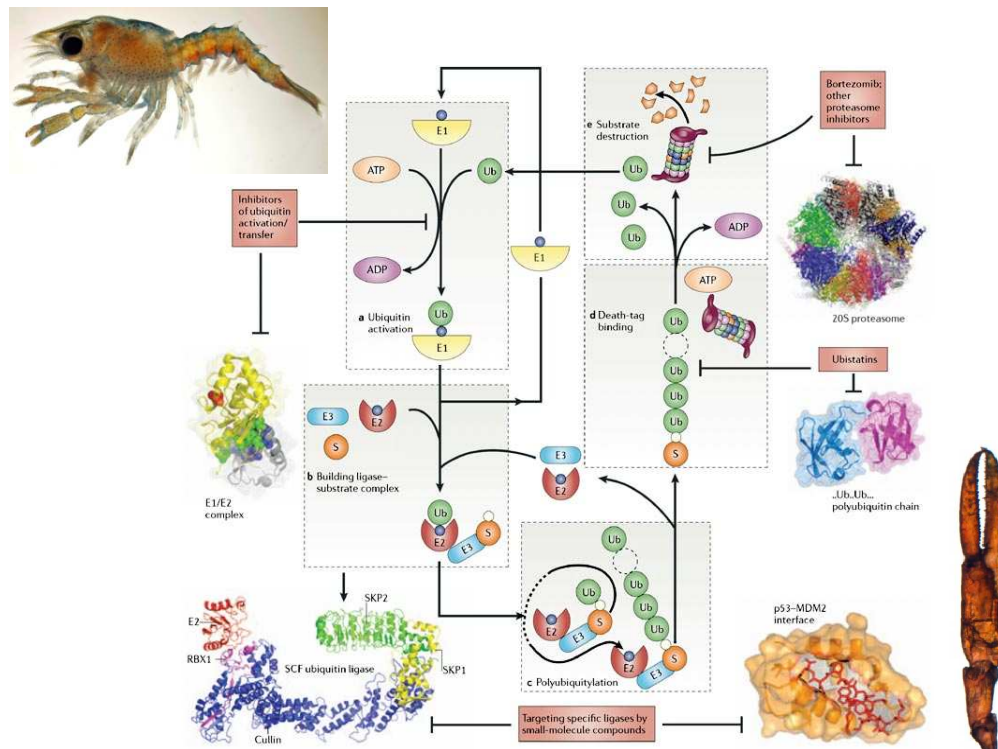


# The role of the proteasome during the larval development of the clawed lobster, *H. gammarus*



## Bachelor Thesis

Philipps-University Marburg,  
Faculty of Biology

In cooperation with the Alfred-Wegener Institute  
for Polar- and Marine Research  
Functional Ecology

Submitted by: Sandra Götze  
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## **Declaration of Authorship**

I hereby declare that this is my own work and effort. This work has not been submitted anywhere for any award. Where other sources of information have been used, they have been acknowledged.

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Bremerhaven, d. 6. Juli 2010

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## Summary

The proteasome is a highly conserved protein complex. It is present in pro- as well as in eukaryotes where it plays an essential role in intra-cellular protein degradation processes. Moreover, it is involved in molt-induced claw muscle atrophy of decapod crustaceans. In this study it was investigated whether the proteasome is also involved in the frequent and consecutive molting processes which lobster larvae pass through during their early development.

The claw muscle tissues of pre-, inter-, and post-molt individuals, staged from Zoea 1 to Zoea 3, and the first juvenile stage were analyzed. The trypsin-like, chymotrypsin-like, and peptidylglutamyl peptidase hydrolase activities of the 20S proteasome were determined. Furthermore, the protein patterns of the four inter-molt stages were separated through 2D-Gel-Electrophoresis. Since proteasomal activity is also related to the expression of ubiquitin, the amount of ubiquitin-mRNA was quantified by Real-Time PCR.

The proteasomal activities of all catalytic sites increased continuously within the first larval stage (Zoea1). In the subsequent two larval stages the proteasome activities increased towards molt but were reduced in the inter-molt phases. Moreover, the chymotrypsin-like site which is the limiting and controlling step in protein degradation seems to gain importance within the zoea 2 and 3 larvae. The chymotrypsin-like site showed increasing activities in the zoea 2 and zoea 3 stages whereas the two other sites did not change. This implies that especially the second and third larval stages have to cope with cellular processes in which a high proteasome attendance is required. After passing the larval stages the activity decreased significantly in the inter-molt juveniles. Moreover, the ubiquitin mRNA quantities changed not significantly throughout the first two larval stages. The zoea 3 was the first developmental stage in which a molt-related change of the quantity could be detected.

I conclude that the proteasome plays an important role in the early development of European lobsters mainly by a) a general stimulation of all three proteasomal activity sites, b) an increase of the rate controlling chymotrypsin-like activity c) and the shift in the ratio between trypsin- like and chymotrypsin like activities. The latter may be paralleled by a change of the proteasomal subunit composition. The concerted action of the proteasomal sites and the “fine-tuning” in between the sides helps to successfully facilitate the rapidly repeating molt processes during the larval development of the lobsters.

## List of abbreviations

2D-PAGE	Two-Dimensional Gel Electrophoresis
AMC	7-Amino-4-methylcoumarin
ANOVA	Analysis of Variation
CHY	Chymotrypsin-like activity of the proteasome
Ct	Cycle number in which the fluorescence of the amplificate exceeds the threshold value of the background fluorescence
DMSO	Dimethyl Sulfoxide
IEF	Isoelectric Focusing
IPG-DALT	Immobilized pH Gradient
Juv 1 (1)	Juvenil 1, post-molt animal
Juv 1 (2)	Juvenil 1, inter-molt animal
PCR	Polymerase Chain Reaction
PGPH	Peptidylglutamyl peptide hydrolase activity of the proteasome
qRT-PCR	quantitative Real-Time Polymerase Chain Reaction
SEM	Standard error of the mean
TRY	Trypsin-like activity of the proteasome
Z1 (1)	Zoea 1, post-molt animal
Z1 (2)	Zoea 1, inter-molt animal
Z1 (3)	Zoea 1, pre-molt animal
Z2 (1)	Zoea 2, post-molt animal
Z2 (2)	Zoea 2, inter-molt animal
Z2 (3)	Zoea 2, pre-molt animal
Z3 (1)	Zoea 3, post-molt animal
Z3 (2)	Zoea 3, inter-molt animal
Z3 (3)	Zoea 3, pre-molt animal



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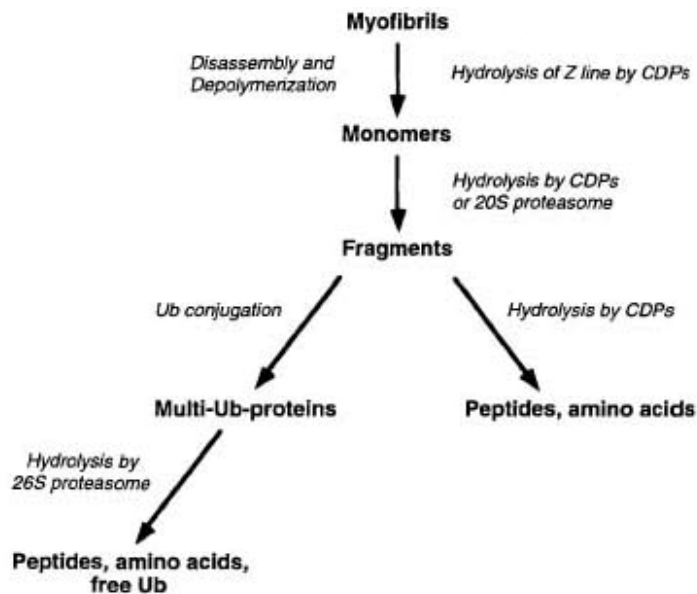
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## 1. Introduction

Different to animals with an endoskeleton, arthropods and, particularly, crustaceans, which possess an exoskeleton, need to molt when they grow. This entails that they have to reduce muscle tissue to slide out of their old exoskeleton. In crustaceans mold-induced muscle atrophy is most distinct in the large claws (Mykles 1999a). To be able to pass the claw muscle through the narrow joints a reduction of 40 % to 75 % of the muscle tissue is necessary (Skinner 1966, Shean and Mykles 1995). Although the muscle is weakened the contractile function remains. This is due to the fact, that the muscle fibers and myofibrils are less degraded, but instead the filament packing is compressed. In detail, ratios of thin to thick myofilaments decrease from 9:1 to 6:1 (Mykles and Skinner 1981). This reduction and re-organization is accompanied by a drastically increase of protein synthesis (e.g. El Haj et al. 1996). The high protein turnover rate seems to be necessary to perform the complex remodeling process.

Two different but complementary proteolytic systems are reported to be mainly responsible for muscle degradation: the calcium-dependent proteases and the ATP/ubiquitin-dependent 26S proteasome (Fig.1.1; Mykles 1990, Mykles and Haire 1991, Mykles 1999a).

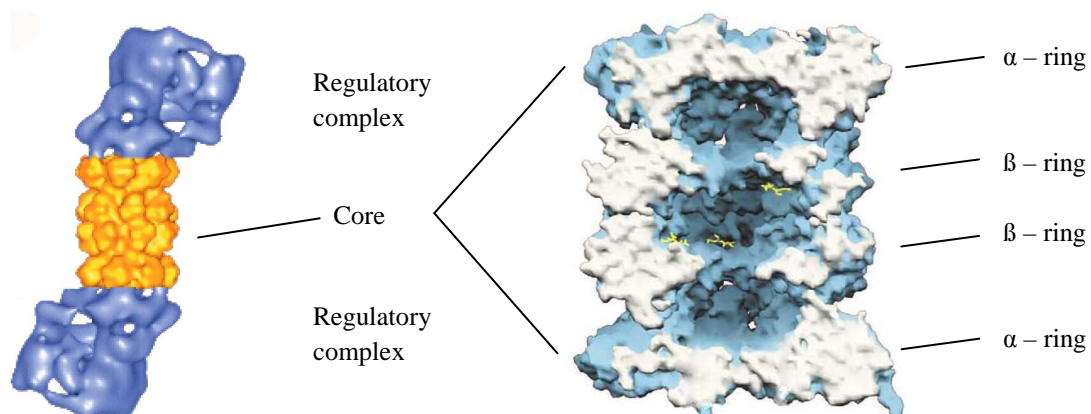


**Fig. 1.1 Pathway for the degradation of myofibrillar proteins:** Illustrated is the predicted involvement of both proteolytic systems during muscle degradation (Source: Mykles 1999a)

## 1.1 The proteasome

The proteasome is a highly conserved multi-catalytic protease complex which is present in prokaryotes as well as in all tissues of eukaryotes (Baumeister et al.1998, De Mot 1999). Since the majority of redundant proteins are degraded via the ATP/ubiquitin-dependent proteasomal pathway, the enzyme seems to hold a regulatory key position in the control of the cell cycle (Hochstrasser 1995). It is involved in cell differentiation, apoptosis, antigen processing, or signal transduction (detailed overview given by Voges et al. 1999). In eukaryotes two forms of proteasome exist: the 20S proteasome (~700 kDa, also called core complex) and the 26S proteasome (~2.5 MDa), whereas only the 20S proteasome is catalytically active. The 26S proteasome consists of the 20S proteasome and additional regulatory subunits. The 26S proteasome performs *in vivo* the ATP/ubiquitin-dependent proteasomal pathway.

The 20S core complex (~700 kDa) is made up of 28 subunits which are organized in four stacked rings (Fig.1.2). The two outer rings consist of seven different  $\alpha$ -subunits and they generate a pore on each site which only unfolded proteins or shorter polypeptides can pass. The two inner rings consist of seven different  $\beta$ -subunits and only they built up the hydrolytic chamber. However, only three subunits of each  $\beta$ -ring are proteolytically active; the sites are named after their preferred cleaving mechanism. The trypsin-like site ( $\beta$ 2) cleaves preferentially after basic residues, the chymotrypsin-like site ( $\beta$ 5) after large hydrophobic residues and the peptidyl-glutamyl-peptidase hydrolase (PGPH) like sites ( $\beta$ 1) after acidic residues.



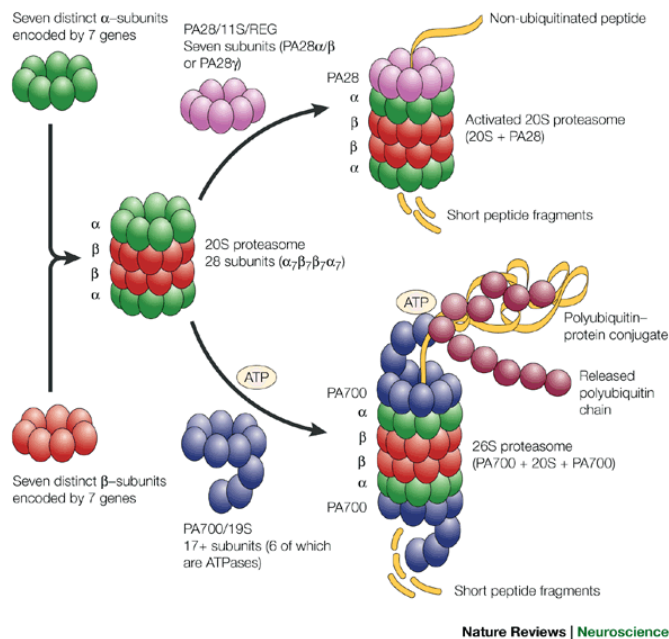
**Fig.1.2 Structure of the eukaryotic proteasome:** the 26S proteasome is illustrated on the left (Source: [Alberts et al. 2005](#)) with the core complex in between the two regulatory complexes. The Core complex is shown enlarged on the right (Source: [Groll et al. 2003](#)). Here the organization of the 4 rings is shown and the localization of the three catalytic sites is marked in yellow.

Recent studies suggest that the PGPH like site should more correctly be termed as caspase like site since it cleaves aspartyl residues much better than glutamyl residues (Kisselev et al. 1999). In this study the active site will still be termed as PGPH-like according to the substrate (a glutamate containing substrate) which was used to assay this activity.

The subunit composition of the proteasome is reported to be extremely variable, depending on e.g. post-translational modifications, on the tissues, or age (Frisan et al. 1998, Dahlmann et al. 2000, Husom et al. 2003). Depending on the composition the proteasome displays different catalytic cleavage preferences.

A certain regulation of the 20S proteasome is already achieved through the compartmentalization of the complex. The pore size which is formed by the  $\alpha$ -subunits restricts the access of proteins into the inner catalytic chamber. However, this feature alone would not be sufficient for the precise regulation of proteasomal activity in vivo. Therefore, the 20S proteasome is mainly attached to several regulatory complexes which inhibit, activate or modulate the proteasomal activity (Baumeister et al. 1998, review: Glickman et al. 2001).

Two of the most important regulatory complexes will be mentioned: the ATP-dependent PA700-complex, which mediates the degradation of ubiquitinated substrates, and the endogenous regulatory complex PA28, which generally activates the proteasome (Fig. 1.3).



**Fig.1.3 Assembly of the 20S proteasome with regulatory complexes:** On the left the assembly of the 4 rings to the 20S proteasome is illustrated. In one case the PA28 complex binds to the outer  $\alpha$ -subunit ring and enhances the degradation of non-ubiquitinated peptides. In the other case the PA700 complex binds and initiate the degradation of polyubiquitin-protein conjugates. (Source: Kevin St P. McNaught et.al. 2001)

## 1.2. Ubiquitin and protein degradation

The main feature of the efficient and highly selective degradation processes is the precise labeling of proteins that are selected for degradation. The majority of these proteins are labeled with ubiquitin and, therefore, degraded via the ATP/ubiquitin-dependent proteasomal pathway. Ubiquitin is a small, 76 amino-acids containing polypeptide. In a complex process it is reversibly attached to protein selected for degradation (in detail described in: [Hochstrasser 1995](#), [Hershko et al.1998](#)). The ubiquitin-protein conjugates are recognized by the PA700-complex, bound to it, and unfolded. The ubiquitin is released and recycled again while the unfolded polypeptide is directed into the catalytic chamber to be degraded.

An elevated protein turnover entails increasing concentrations of ubiquitin-protein conjugates. This, in turn, implies increasing concentrations of ubiquitin. Several studies investigating the gene expression during the molt cycle of crustacean, found a molt-dependent ubiquitin mRNA expression in the claw muscle tissue ([Shean et al. 1995](#), [Koenders et al. 2002](#), [Spees et al. 2003](#)). The ubiquitin expression showed great variations. Shortly before the molt of the animals the expression of ubiquitin mRNA significantly increased. In contrast, inter-molt lobster possessed a remarkably lower content of ubiquitin. These results indicate a close connection between the level of protein degradation, the level of ubiquitin mRNA expression, and the activity of the 26S proteasome.

## 1.3. Larval development of the European lobster, *H.gammarus*

Female lobsters carry a clutch of fertilized eggs attached to the pleopods on the ventral site of their abdomen. The embryonic development lasts around one year before the larvae hatch as zoea 1 stage. The zoea 1 molts into two subsequent larval stages, zoea 2 and zoea 3 (all three stages are illustrated in **Fig.1.4**).



**Fig.1.4** Zoea 1, zoea 2 and zoea 3 (from left to right): The stages are not illustrated in proportional size to each other

The zoea stages are reported to be planktonic and carnivore (Charmantier et al. 1991). The next molt is accompanied with a metamorphosis-like transition into juvenile lobsters (Fig.1.5). These early juveniles look more like adult lobsters and soon they start to settle for a benthic lifestyle. The next stages mainly molt in order to grow and to become adult.



**Fig. 1.5** The first juvenile stage

The larval development takes on average 20 days. The duration mainly depends on the water temperature (Schmalenbach et al. 2010). From hatching to the first juvenile stage the larvae undergo several anatomical-morphological and physiological changes (reviewed by Charmantier et al. 1991). Most distinct differences appear with the molt into the juvenile stage. For instance, there is a change in the general body shape, a variation in the total digestive enzyme activities, or a change in behavior (Charmantier et al. 1991). Moreover, the claws become symmetric and increase significantly in size (Fig.1.6).



**Fig.1.6** Claw development and claw growth from the Zoea 1 stage to the juvenile lobster

### 1.5. Objectives of this study

It is not known to date whether the same mechanisms are involved in molt and metamorphosis of larval crustaceans as in the adults. The early developmental stages are subjected to immense changes and rapidly repeating molt cycles. Their claw anatomy indicates that the molt is a similarly critical moment as it is for the adult lobsters. The claws are broader and thicker than the narrow joint between the coxa and the basis which means that the lobster larvae have to reduce or re-organize muscle tissue for successful molts.

Therefore, I raise the hypothesis that the 20S proteasome is already involved in the molts between the larval stages. Accordingly, I want to investigate whether the proteasomal activities change during the development of the European lobster (*H.gammarus*) and, particularly, within the molt cycles of the individuals. The proteasomal activity in the crude claw extracts will be verified through the highly specific inhibitor epoxomycin ([Meng et al. 1999](#), [Kisselev et al. 2001](#)) and the endogenous activator complex PA28 ([Mykles 1996](#)).

Furthermore I will investigate whether changes are accompanied by variations in the subunit patterns. The expression of ubiquitin seems to be closely correlated to the molt stage and the proteasomal activity. I want to finish this study with the investigation whether already during the developmental stages the ubiquitin expression displays a molt-dependent cycle. All parts of my thesis will lead to a detailed knowledge about the involvement of the proteasome during early stages of the European lobster, and moreover will help for the better understanding of larval development and those factors which influence molt success.

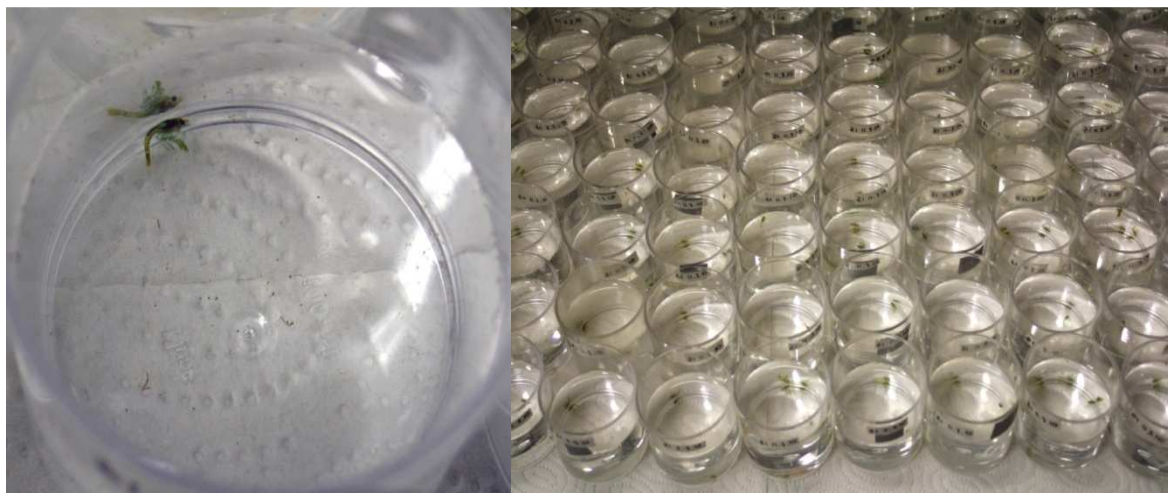


## 2. Materials and Methods

### 2.1. Experimental design

#### 2.1.1 Maintenance and rearing of larvae

Lobster larvae of all stages (zoea1 to zoea 3) as well as the first juvenile stage (juv 1) were obtained from the lobster rearing facility of the Marine Station Helgoland and shipped to the laboratories in Bremerhaven. Immediately after arrival the larvae were separated and maintained individually in 100 ml beakers filled with natural seawater (Fig.2.1). The water temperature ranged between 17 °C and 19 °C. Once per day the seawater was exchanged and the larvae were fed with freshly hatched nauplii of the brine shrimp *Artemia salina* (Leach 1819).



**Fig.2.1: Maintenance and rearing of lobster larvae: a) Single beaker containing one larva b) Overview on rearing assembly**

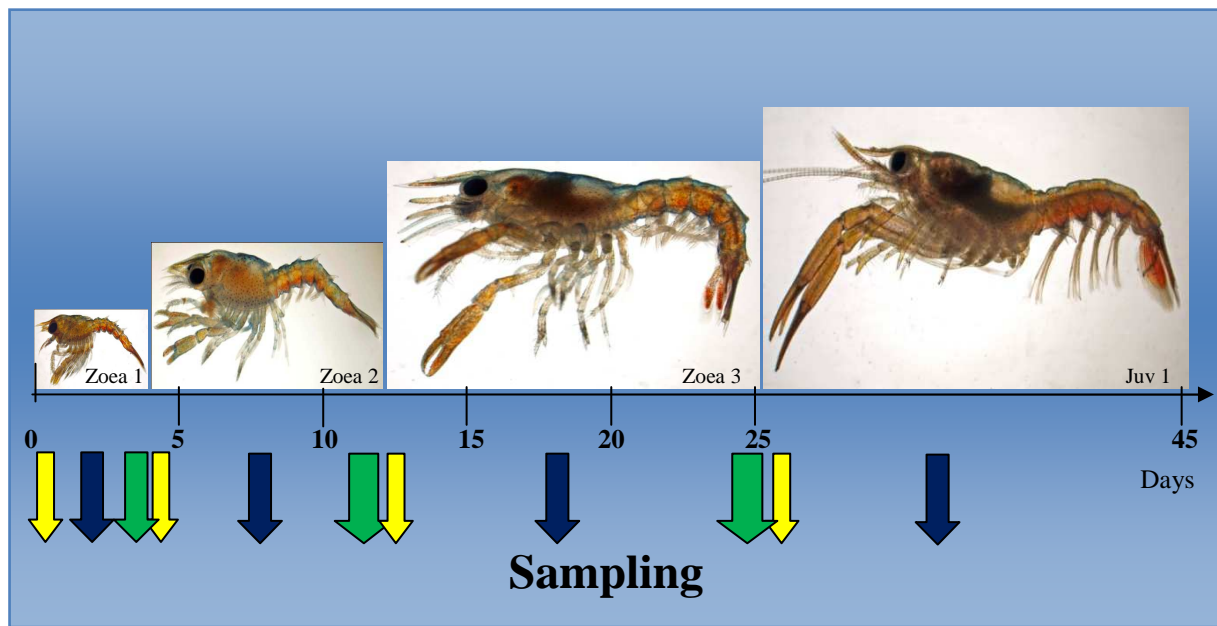
One group of larvae (n = 25) was allowed to grow up until they reached the juvenile stage. This approach was carried out to determine the duration of the larval stages at the given experimental conditions (Table 2.1). Additional data about the duration of each larval stage were provided by [Schmalenbach & Franke \(2010\)](#). The mortality of the larvae was less than 15 %.

**Tab.2.1:** Overview over the time periods the larvae needed to devolve into the next stage (on the left) and sampling after hatch or molt, respectively, of each larval and juvenile stage (Means  $\pm$  S.D, n = 15).

	Stage Duration (Days)	Molt stage		
		Hatched/post-molt	Inter-molt	Pre-molt
		Time of sampling	Time of sampling	Time of sampling
<b>Zoea 1</b>	<b>4.33 <math>\pm</math> 0.8</b>	Max. 12 h after hatch	3.0 $\pm$ 0.5	$\pm$ 1.0 before molt
<b>Zoea 2</b>	<b>8.13 <math>\pm</math> 2.4</b>	Max. 12 h after molt	3.0 $\pm$ 0.5	$\pm$ 1.0 before molt
<b>Zoea 3</b>	<b>12.80 <math>\pm</math> 1.1</b>	Max. 12 h after molt	5.5 $\pm$ 0.5	$\pm$ 1.0 before molt
<b>Juvenil</b>	<b>20.33 <math>\pm</math> 3.2</b>	Max. 12 h after molt	10.0 $\pm$ 0.5	-

The samples were taken after molting (post-molt), during the inter-molt phase (inter-molt) and before the next molt (pre-molt) of each stage. A detailed schedule illustrating the molt intervals and the sampling strategy is shown in Fig. 2.2.

Zoea 1 larvae were sampled after hatching (Z1(1)) to obtain the post-molt individuals. When the developmental stages were unknown, the larvae were first raised until they molted into the subsequent stage.



**Fig.2.2: Stage duration of the lobster larvae and sampling intervals:** The yellow arrows indicate the time periods when the post-molt individuals were sampled, the blue arrows indicate the sampling of the inter-molt individuals and the green arrow indicates the sampling of the pre-molt individuals.

The post-molt individuals were sampled not more than 12 hours after hatching or molting, but preferably as close after ecdysis as possible. The inter-molt individuals were sampled after they passed half of the predicted duration of the stage. In order to sample the pre-molt animals

in due time the larvae of the respective batch were controlled for the first larvae to molt into the next stage. When this happened, the remaining larvae were shortly before the molt as well and, consequently, they were also sampled for analysis.

### **2.1.2 Sampling and preparation of tissue extracts for enzyme assays and 2D-electrophoresis (IPG-DALT)**

From each molting stage the claws of 25 larvae were sampled for enzyme assays and 2D-electrophoresis (Görg et al. 2004). From each larva both claws were completely excised, briefly blotted dry on filter paper, and transferred into a reaction tube. The claws were weighed and homogenized in 45 µl of homogenizing buffer using a micro-pestle (detailed buffer compositions are given in the appendix). Subsequently, the extracts were centrifuged for 10 min at 9000 g and 4 °C (Eppendorf centrifuge, 5417R) and aliquoted into two samples of 20 µl each. All samples were stored at -80 °C until further analysis.

### **2.1.3 Samples for the quantitative Real-Time PCR (qRT PCR)**

Ten larvae from each molting stage were pooled to provide one sample for qRT PCR analysis. In detail, 10 whole larvae of the same molt stage were transferred into one reaction tube containing RNA-later reagent (*Ambion, AM7024*). The reagent was allowed to penetrate into the tissue overnight at room temperature. The next day the tubes were stored at -80 °C until further analysis.

## **2.2. Proteasomal activities**

The proteasomal activities were investigated in claw muscle extracts. Ten lobster larvae from each molt stage were analyzed.

The trypsin-like, the chymotrypsin-like, and the peptidylglutamyl peptide hydrolase activity (PGPH) of the 20S proteasome were assayed by using the fluorogenic substrates *Boc-Leu-Arg-Arg-AMC* (PeptaNova, 3140), *Suc-Leu-Leu-Val-Tyr-AMC* (Enzo Life Sciences, P-802) and *Z-Leu-Leu-Glu-AMC* (*Enzo Life Sciences, 9345*). Additionally, the trypsin-like and the chymotrypsin-like activities were verified by inhibition of the enzyme with the highly specific inhibitor epoxomicin (*PeptaNova, 4381-0.02*). The PGPH activity was not inhibited but,

instead, activated by the endogenous regulatory complex PA28 (11S regulatory subunit, *Enzo Life Sciences*, 9420-0025).

Prior to routine analysis, Michaelis-Menten kinetics were performed for the three proteasomal active sites to determine suitable substrate concentrations in the final enzyme assays. The sensitivity of the proteasome against epoxomicin as well as its specificity was examined with adult lobster samples. These assays were performed with crude claw homogenate samples from adult European lobsters.

The specificity of the inhibitor epoxomicin was analyzed by comparing the proteasomal activities with digestive enzymes from the gastric fluid of adult lobsters. The gastric fluid is rich in digestive enzymes like trypsin, chymotrypsin, or other proteases but does not contain proteasome. Digestive trypsin and chymotrypsin are capable of degrading the same fluorogenic substrates as the proteasome. However, in contrast to the proteasome, the gastric enzymes should neither be affected by the inhibitor epoxomicin nor by the activator PA28.

### **2.2.1 Enzyme assays with the NanoDrop device**

Since measuring fluorescent signals with the NanoDrop device requires only 2  $\mu\text{l}$  of reaction mixture, the total volume per assay was reduced adequately. All reaction mixtures had a total volume of 25  $\mu\text{l}$ ; containing assay buffer, sample, solvent, substrate and, in case, other reagents. Except the PA28 which already was delivered in solution, all substrates were dissolved in dimethyl sulfoxide (DMSO) and prepared as 10- or 20-fold stock solutions freshly before the measurements.

The reactions were started in ice cooled reaction tubes containing assay buffer and the samples with the addition of the substrate. The tubes were immediately transferred into a thermomixer and incubated for 1 h at 37 °C. The released fluorescence was measured exactly after this hour. For each substrate a blank was run to control whether the substrate was degraded within the incubation. These approaches contained only buffer and the substrate but no enzyme. The released fluorescence was detected at 365 nm (excitation) and 437 nm (emission) with a NanoDrop device (*PeQLab, NanoDrop 3300 v.2.7.0*).

### 2.2.2 Michaelis-Menten kinetics

The substrate concentrations for measuring the trypsin-like and the PGPH-activity ranged from  $0.05 \text{ mmol}\cdot\text{L}^{-1}$  to  $2 \text{ mmol}\cdot\text{L}^{-1}$ , and for the chymotrypsin-like activity from  $0.05 \text{ mmol}\cdot\text{L}^{-1}$  to  $1.5 \text{ mmol}\cdot\text{L}^{-1}$ .

The enzyme activities were determined for different substrate concentration in triplicate and paralleled by a control (blank) without enzyme. The blank values were subtracted from the sample values. The results were quantified by applying a calibration curve with 7-Amino-4-methylcoumarin (AMC, see 2.2.5.1). The Michaelis-Menten constants ( $K_m$ ) were calculated with the program GraphPad Prism 5 (GraphPad Software, Version 5.02).

### 2.2.3 Inhibition and activation of the 20S proteasome

#### “Sensitivity”

The applied concentrations of epoxomycin ranged for the chymotrypsin-like activity from  $2.5 \text{ }\mu\text{mol}\cdot\text{L}^{-1}$  to  $50 \text{ }\mu\text{mol}\cdot\text{L}^{-1}$  and for the trypsin-like activity from  $10 \text{ }\mu\text{mol}\cdot\text{L}^{-1}$  to  $100 \text{ }\mu\text{mol}\cdot\text{L}^{-1}$ . Blanks were run to determine the auto-fluorescence of the substrate and of the inhibitor, respectively. Additionally, one approach served as control for the level of uninhibited activity. A detailed list of the components of reaction mixture is given in Table 2.2.

#### “Specificity”

The gastric fluids of 3 adult European lobsters were obtained directly from the stomach with a syringe connected to a plastic tube. The samples were centrifuged for 15 min at 13000 g and  $4 \text{ }^\circ\text{C}$  to remove food residues. The supernatants were diluted 1:20 with distilled water before they were applied to reaction tubes. A detailed list of the components is also given in Table 2.2.

First, the trypsin and the chymotrypsin activities of the gastric fluids were determined to obtain the unaffected level of activity. Concurrently, assays were run with epoxomycin. For the trypsin-like activity a stock solution of  $1 \text{ mmol}\cdot\text{L}^{-1}$  epoxomycin was prepared, and for the chymotrypsin-like activity a stock solution of  $100 \text{ }\mu\text{mol}\cdot\text{L}^{-1}$ . Additionally it was investigated

whether the gastric enzymes are able to degrade the PGPH-substrate and whether the activity can be enhanced by PA28. The PA28 concentration amounted to 4  $\mu\text{g}\cdot\text{ml}^{-1}$ .

**Table 2.2: Detailed compositions of the reaction mixtures:** measuring the Michaelis-Menten kinetics, the inhibition assays for detecting the sensitivity of epoxomycin and the specificity of epoxomycin and PA28.

	Michaelis- Menten kinetics	“Sensitivity”	“Specificity” Control	“Specificity” Treated	Blanks
Assay buffer	20.00 $\mu\text{l}$	16.50 $\mu\text{l}$	20.00 $\mu\text{l}$	18.75/(18.00) $\mu\text{l}$	22.50 $\mu\text{l}$
Sample	2.50 $\mu\text{l}$	5.00 $\mu\text{l}$	2.5 $\mu\text{l}$	2.50 $\mu\text{l}$	-
Epoxomycin	-	1.00 $\mu\text{l}$	-	1.25 $\mu\text{l}/(-)$	-
PA28	-	-	-	-/(2.00 $\mu\text{l}$ )	-
Substrate	2.50 $\mu\text{l}^1$	2.50 $\mu\text{l}^1$	2.50 $\mu\text{l}^1$	2.50 $\mu\text{l}^1$	2.50 $\mu\text{l}^1$

<sup>1</sup> stock solutions of 1mmol·L<sup>-1</sup>

## 2.2.4 Measuring larval activities

The concentrations of the substrates, the epoxomycin, and the PA28 for measuring larval proteasome activities are listed in Table 2.3.

**Tab.2.3: Overview over the substrates and their concentrations used in the different enzyme assays**

	Substrate	concentration in the assay
Trypsin-like activity	<i>Boc-Leu-Arg-Arg-AMC</i>	0.75 mmol · L <sup>-1</sup>
Chymotrypsin-like	<i>Suc-Leu-Leu-Val-Tyr-AMC</i>	0.50 mmol · L <sup>-1</sup>
PGPH-like activity	<i>Z-Leu-Leu-Glu-AMC</i>	1.00 mmol · L <sup>-1</sup>
Inhibitor	<i>Epoxomycin</i>	2.50 $\mu\text{mol L}^{-1}/100 \text{ mmol L}^{-1}$
Activator	PA28	0.05 $\mu\text{g}/\mu\text{l}$

A detailed schedule of reaction mixture components in the different assays are given in Table 2.4.

**Table 2.4: Detailed outline of the compounds of the reaction mixtures:** measuring the untreated and the treated trypsin-like the chymotrypsin-like and the PGPH activities.

	PGPH activity	assays	(TRY and CHY)	(PGPH)	assays
Assay	17.50 $\mu\text{l}$	22.50 $\mu\text{l}$	17.50 $\mu\text{l}$	17.50 $\mu\text{l}$	21.25 $\mu\text{l}$
Sample	5.00 $\mu\text{l}$	-	5.00 $\mu\text{l}$	5.00 $\mu\text{l}$	-
DMSO	1.25 $\mu\text{l}$	-	-	-	-
Epoxomycin	-	-	1.25 $\mu\text{l}$	-	-/(1.25 $\mu\text{l}$ )
PA28	-	-	-	1.25 $\mu\text{l}$	1.25 $\mu\text{l}/(-)$
Substrate	1.25 $\mu\text{l}^2$	2.50 $\mu\text{l}^1$	1.25 $\mu\text{l}^2$	1.25 $\mu\text{l}^2$	2.50 $\mu\text{l}^1$

<sup>1</sup> of a 10-fold stock solution; <sup>2</sup> of a 20-fold stock solution

### 2.2.5. Analysis of data

The relative fluorescence (RF) obtained from the enzymatic measurements was quantified and expressed in relation to the protein content of the sample as  $\text{Units} \cdot \text{g}_{\text{Prt}}^{-1}$  ( $1\text{U} = \mu\text{mmol} \cdot \text{min}^{-1}$ ). The protein content of the sample was determined after [Bradford \(1976\)](#). A standard curve with the product 7-amino-4-methylcoumarin (AMC) was established to calculate product concentrations from RF units.

#### 2.2.5.1 Calibration curve of 7-Amino-4-methylcoumarin (AMC)

A calibration curve was established with 7-Amino-4-methylcoumarin (AMC, *Fluka, 08440*). The fluorescence of six concentrations, ranging from  $0.025 \mu\text{mol} \cdot \text{L}^{-1}$  to  $10 \mu\text{mol} \cdot \text{L}^{-1}$  were assayed. The AMC substrate was dissolved in DMSO and prepared as 10-fold stock solution for each concentration. Then,  $2.5 \mu\text{l}$  of the particular concentration were added to  $22.5 \mu\text{l}$  of assay buffer and the fluorescence was measured with the NanoDrop device. Each concentration was measured in 5 parallels of which a calibration curve was calculated.

#### 2.2.5.2 Protein quantification after Bradford

The protein quantification after [Bradford \(1976\)](#) was done with a commercial protein dye reagent (*Biorad, 500-0006*). The reagent was diluted 1:5 with distilled water before use. Bovine serum albumin (*Biorad, 76290A*) was used as protein standard.

Increasing amounts of the standard, containing  $0 \mu\text{g}$  to  $5 \mu\text{g}$  of protein, were applied in duplicate onto a 96-well plate and filled up with distilled water to  $25 \mu\text{l}$ . Samples ( $5 \mu\text{l}$ ) were also applied in duplicate and filled up to  $25 \mu\text{l}$ . The reactions were started with addition of  $125 \mu\text{l}$  of the dye reagent. After 5 min of incubation the optical density was measured at 600 nm with a microplate reader (*Thermo Scientific, Multiscan*).

#### 2.2.5.3 Calculations

The mean RFU values measured after 1 hour with the NanoDrop device were quantified by using a calibration curve with the product AMC.



The calibration curve with AMC followed a first-order linear regression:

$$y = 3702.4x + 185.31$$

$$y = \text{mean RFU} \cdot \text{h}^{-1}$$

$$x = \text{resulting product in } \text{pmol} \cdot \mu\text{l}^{-1} \cdot \text{h}^{-1}$$

The resulting product was calculated as pmol product generated within 25  $\mu\text{l}$  assay volume per minute.

$$x (\text{picomol} \cdot 25 \mu\text{l}^{-1} \cdot \text{min}^{-1}) = \frac{\left[ \frac{(\text{Mean RFU} - 185.31)}{3702.4} \right]}{60} * 25$$

Finally, these values were related to the amount of protein in each assay and recalculated for the product (in  $\mu\text{mol}$ ) which was generated per g protein and per minute. The results of every molt stage were averaged and the standard error of the mean was calculated with the program GraphPad Prism 5 (GraphPad Software, Version 5.02).

The results obtained from the inhibited and activated assays were compared with the results of the untreated assays and expressed as per cent of the remaining activity or the enhanced activity, respectively. The average values of the untreated assays were always set as 100 %.

#### 2.2.6.4 Graphs and Statistics

The enzyme activities were expressed as average specific activities ( $\text{U} \cdot \text{g}_{\text{Prt}}^{-1}$ ) and data sets were analyzed by a One-way ANOVA followed by a Tukey HSD test. The statistical analyses were carried out with the program Statistica 7.1 (Systat Software, Inc.). Significant differences between the stages were indicated by different letters when p-values  $\leq 0.05$ . If not stated otherwise the graphs were created with the program SigmaPlot 10.0 (Systat Software, Inc.).



## 2.3. Quantitative Real-time PCR

### 2.3.1 Sample preparation

The total RNA required for the quantitative RT-PCR was isolated from a pool of 10 claws from each sampling stage (Z1 (1) to Juv. 1(2)) with the RNeasy Mini Kit (*Qiagen, 74104*). All reagents were provided in the kit.

The claws were removed from the larvae and transferred into new reaction tubes already containing 350  $\mu$ l of RLT buffer. The tissues were homogenized with a micro-pestle and the extracts were centrifuged for 3 min at 13000 g and 4 °C. Thereafter, the supernatants were transferred into new reaction tubes and 350  $\mu$ l of ethanol (70 %) were added. The solutions were mixed thoroughly and 700  $\mu$ l of the mixture were applied onto RNeasy mini columns which, again, were placed in 2 ml collection tubes.

The tubes were centrifuged for 15 s at 10 000 g and the eluents were discarded. Then, 700  $\mu$ l of RW1 buffer were applied onto the columns and again centrifuged under the same conditions. The collection tubes, containing the eluents, were discarded. The columns were transferred into new collection tubes and 500  $\mu$ l RPE buffer were applied onto the column. This was followed by another centrifugation step for 15 s and the eluents were discarded again. Another 500  $\mu$ l RPE buffer were applied onto the columns. The following centrifugation step lasted 2 min at 10000 g to dry the RNeasy silica-membranes. The columns were transferred into new collection tubes and the bound RNA was eluted with 30  $\mu$ l RNase-free water during another centrifugation step for 1 min at 10000 g. To increase the RNA yield, a second elution step was done. Therefore, the 30  $\mu$ l of the eluents were again applied onto the columns and centrifuged under the same conditions. The RNA concentrations were determined with a NanoDrop 1000 (*PeQLab, NanoDrop 1000, Version 3.2*).

Prior to cDNA synthesis, the total RNA samples were subjected to a DNase digest with the Turbo DNA-free Kit (*Ambion, AM1907*). This approach contained 10  $\mu$ g of total RNA, 5  $\mu$ l of 10- fold buffer, and 1  $\mu$ l of DNase, filled up to 50  $\mu$ l with RNase free water. The mixtures were incubated for 30 min at 37 °C before 5  $\mu$ l of DNase inactivation solution were added. Now, the tubes were vortexed and incubated at room temperature for another 5 min. Contaminating DNA was precipitated in the followed centrifugation step at 13000 g for 2 min. The supernatants were transferred into new reaction tubes and the amount of RNA was measured with a NanoDrop device. The samples were stored at -80 °C.

### 2.3.2 cDNA-Synthesis

The total RNA which is subjected to the reverse transcription should amount to 0.4 µg in each sample. The calculated sample volumes which were needed for the cDNA synthesis, but at maximum 9.9 µl, were then transferred into PCR reaction tubes, incubated for 10 min at 70 °C and stored on ice. Meanwhile, the master mix for the reverse transcription was prepared (detailed composition in Tab.2.5). All components were included in a High-Capacity cDNA RT Kit (*Applied Biosystems, 4368814*).

**Table 2.5: Composition of the master mix used for the cDNA synthesis**

Components of the master mix	Per reaction (20 µl)
10X RT-Buffer	2.0 µl
dNTP-Mix (25X, 100mM)	0.8 µl
RT Random Primers (10-fold)	2.0 µl
MultiScribe Reverse Transcriptase	1.0 µl
RNase free water	4.2 µl
Total	10.0 µl

Ten µl of the master mix were added to each sample. If the sample volume was less than 9.9 µl, it was filled up to 20 µl with RNase free water. The reverse transcription of the isolated RNA into first-strand cDNA was carried out in a thermocycler (*Eppendorf, Mastercycler pro, Vapo protect*). In the first step the samples were incubated for 19 min at 25 °C and then the temperature was raised to 37 °C. At this temperature the samples were incubated for another 120 min. In the last step they were heated up to 85 °C for 5 s. The cDNA was stored at -80 °C until further use.

### 2.3.3 Primers

Besides the target gene, the ubiquitin cDNA, it is also necessary to amplify housekeeping genes. The expression of these genes remains constant irrespective of the treatment. Therefore, they can be used to normalize the amplification rate of the target gene. Alpha actin, beta-actin and the sodium-potassium ATPase  $\alpha$ -subunit of *H.gammarus* were chosen as suitable housekeeping genes.

The DNA sequence of the ubiquitin from the European lobster (*H.gammarus*) was not available. In order to obtain the nucleotide sequence of the polyubiquitin cDNA, a sequencing PCR was carried out (described in a previous report). The obtained sequence is illustrated in Fig. 2.3.



**Fig. 2.3: Sequencing PCR:** CLUSTAL 2.0.12 alignment of the Ubiquitin sequences from the European lobster and the American lobster

The primers for the quantitative Real-Time PCR were created on the basis of this sequence. The sequences of the housekeeping genes were obtained from data bases of the *National Center for Biotechnology Information* (NCBI). All primers were designed with the software *primer express* (*Applied Biosystems*). For each gene one pair of primers were selected. All primers were purchased from *ThermoScientific*. The detailed primer sequences are listed in Tab.2.6.

**Tab.2.6: Primer sequences used for the qRT-PCR**

<b>Ubiquitin</b>	Sense	5' – ATC CAG AAA GAA TCT ACA CTA CAT TTG G – 3'
	Antisense	5' – TTT GAA CAT TTC ATC CGG AAT ACT T – 3'
<b>Sodium-potassium ATPase <math>\alpha</math>-subunit</b>	Sense	5' – GTG AAC GGC GAT GCT TCA G – 3'
	Antisense	5' – TCG CCA GCC CTT TAC ATC TC – 3'
<b>Beta-Actin</b>	Sense	5' – CGT AAG GAT CTG TAT GCC AAC ACA – 3'
	Antisense	5' – CTG CAT CCT GTC GGC AAT T – 3'
<b>Alpha-Actin</b>	Sense	5' – CCT GCT TCG AAA CCC GCT AA – 3'
	Antisense	5' – AGG AGG CAA CAA GCC ATG TAC T – 3'

All primers were dissolved in TE-buffer to a stock solution of  $100 \mu\text{mol}\cdot\text{L}^{-1}$ . The stock solutions were divided into  $20 \mu\text{l}$  aliquots and stored at  $-20 \text{ }^\circ\text{C}$ . Before the primers were used they were diluted with TE-buffer to a final concentration of  $5 \mu\text{mol}\cdot\text{L}^{-1}$ .

### 2.3.4 Primer and sample evaluation

For the evaluation of the sample and the primers, a cDNA template of the freshly hatched zoea 1 was used. The template was diluted with RNase free water to provide 5 concentrations ranging over 2 log steps of the total RNA (Table 2.7).

**Table 2.7: Sample dilution schedule:** Dilution steps of the zoea 1 cDNA template for running the sample evaluation. Shown are the concentrations in the dilution approaches and the peculiar end concentration per well. Additionally the (-) log step calculated by the input of total RNA is shown

Template	Dilution	ng per approach	-(log) of total RNA
Zoea 1 cDNA	1:20	2.00	3
	1:40	1.00	2,70
	1:100	0.40	2,30
	1:200	0.20	2,00
	1:1000	0.04	1,30

Each template concentration was analyzed in triplicates and for each pair of primers. Additionally to the samples, control approaches were done for each gene. The first control was the “no template control” (NTC) in which the sample was replaced by water. The second control was the “no cDNA control” (-RT) in which the cDNA samples were replaced by the total RNA samples, already applied to the cDNA synthesis. Coincidentally to the dilution series, four master mixes were prepared. They were composed of the SYBR® Green PCR Master Mix (*Applied Biosystems, 4309155*), sense and antisense primers amplifying for one gene and RNase free water. The master mixes were ice cooled and the detailed composition is given in Table 2.8.

**Table 2.8: Composition of the master mix for qRT-PCR**

Components of the master mix	Per reaction (18 $\mu\text{l}$ )
2X SYBR Mix	10.0 $\mu\text{l}$
Sense primers	1.2 $\mu\text{l}$
Antisense primers	1.2 $\mu\text{l}$
RNase free water	4.2 $\mu\text{l}$

A 96 well plate (*Applied Biosystems, MicroAmp® Optical 96-Well Reaction Plate, N8010560*) was loaded with 2µl of samples and controls as shown for the ubiquitin in Fig.2.4.

**Fig.2.4: Layout of the 96 well plate for the ubiquitin:** Two µl of the different sample concentration were applied onto the well plate. To the wells 18 µl of master mix containing the fitting primer pairs were added. Additionally, two control assays were run; the no template control (NTC – containing water) and the no cDNA control (-RT – containing the total RNA sample)

	1	2	3	4	5	6	7	8	9	10	11	12
<b>A</b>	<b>Ubiquitin</b>			<b>Ubiquitin</b>			<b>Ubiquitin</b>			<b>Ubiquitin</b>		
	2 ng	2 ng	2 ng	1 ng	1 ng	1 ng	0.4 ng	0.4 ng	0.4 ng	0.2 ng	0.2 ng	0.2 ng
<b>B</b>	<b>Ubiquitin</b>			<b>Ubiquitin</b>		<b>Ubiquitin</b>						
	0.04 ng	0.04 ng	0.04 ng	NTC	NTC	(-RT)	(-RT)					

The plate was placed on ice and 18 µl of master mix, containing the primers, were added to each well. The plate was covered with an adhesive optical film (*Applied Biosystems, 4360954*) and the Real-Time PCR was carried out at the Real-Time-PCR System (*Applied Biosystems, 7500*). Initially, the samples were incubated at 50 °C for 2 min. Then they were heated up to 95 °C for another 10 min. Subsequently, the PCR passed over to the cyclic phase of heating and cooling. The samples were incubated in each cycle for 15 s at 95 °C and were then cooled down to 60 °C for 1 min. This was repeated 40 times.

Immediately after the PCR run a dissociation curve analysis was started. A temperature ramp was created in which the double-stranded DNA template, produced during the PCR run, was melted again into single stranded DNA. During melting the bound SYBR green is released and the fluorescent signal decreases. The melting point is thereby unique for each DNA template. This allows to control whether only a single or several templates were amplified. Moreover, primer failures like dimmer-forming or inter-specificity can be detected. The conditions for the dissociation curve analysis were composed of an incubation step at 95 °C for 15 s, followed by a 1 min lasting step at 60 °C. Afterward the temperature was continuously increased every 15 s until 95 °C were reached.

### 2.3.5 Measurement of samples

The procedure for analyzing the cDNA templates of all molt stages were mainly the same as described above. All samples were diluted 1:40 with RNase free water. Each sample was analyzed in triplicates and for each pair of primers. NTC and –RT controls were run for each sample and gene.

### 2.3.6 Evaluation of data

Differences in gene expression were calculated with the comparative delta-delta Ct method (pers. com. Dr. Magnus Lucassen, AWI Bremerhaven) and expressed as x-fold change of amplification.

The Ct values of the three parallels measured for each sample and each gene were averaged. Subsequently, the average Ct values were used to calculate the  $\Delta Ct$  - value.

$$\Delta Ct = \text{Average } Ct_{\text{Target}} - \text{average } Ct_{\text{Housekeeping}}$$

The  $\Delta\Delta Ct$  – values were calculated with the freshly hatched Zoea 1 larvae as control group while the other larval and the juvenile stage were defined as treated groups.

$$\Delta\Delta Ct = \Delta Ct_{\text{Treated}} - \Delta Ct_{\text{Control}}$$

In the last step, the x-fold expression of the untreated group to the treated group was calculated as:

$$\text{Ratio} = 2^{-\Delta\Delta Ct}$$

## 2.4. Two-dimensional Gel-Electrophoresis (IPG-DALT)

Samples from the four inter-molt stages were used for analyzing the proteasomal subunit composition by IPG-DALT. First, the proteins were separated by isoelectric focusing (IEF) using Immobiline DryStrips with a pH gradient from pH 3 to pH 10 (*GE Healthcare, 17-6001-11*).

Thirty-five  $\mu\text{l}$  of samples were mixed with 90  $\mu\text{l}$  of rehydration solution (see appendix for detailed buffer composition) according to the specification of the manufacturer. One approach

contained, besides the juvenile lobster sample, 2.5 µl of an internal standard (*BioRad, 2D-SDS-PAGE Standards, 161-0320*).

The total volume (125 µl) was transferred onto the bottom of the dry strip-holders. The cover foils of the IEF-dry strips were carefully removed and the strips were placed, with the gel side downward, into the rehydration solution. The strips were overlaid with ca. 700 µl of dry strip cover fluid and the strip holders were covered with the lid. Then, the strip holders were placed onto the electrode plate of the IPGphor device (Pharmacia Biotech). The power supply was set to 0.05 mA per strip. The voltage conditions were 30 V for 15 h, the 500 V for 30 min, 1000 V for 30 min, and, finally, 8000 V for 1 hour.

The separating gels (T=10 % and C=2.7 %) for the second dimension were prepared according to [Laemmli \(1970\)](#) and are shown in **Tab.2.9**.

**Tab.2.9: Composition of the separating gels**

	<b>Separating gel (ml)</b>
<b>Distilled water</b>	5.000
<b>Stacking gel buffer</b>	3.120
<b>Separating gel buffer</b>	-
<b>Acrylamide (30 %)</b>	4.160
<b>SDS (10 %)</b>	0.125
<b>APS (10 %)</b>	0.063
<b>TEMED</b>	0.010
<b>Total volume</b>	<b>12.500</b>

After the IEF finished, the IPG strips were taken from the strip holders, carefully rinsed with distilled water, and transferred into test tubes containing solution A (detailed buffer composition is given in the appendix). The strips were incubated in this solution for 15 min at room temperature. The buffer A was discarded and buffer B was filled into the test tubes. The strips were incubated in this solution for another 15 min. Then the strips were taken out and carefully, rinsed with distilled water and placed with the anode side to the left onto the separating gel. It was very important to ensure that there was no cleft between the strip and the gel.

Electrophoresis was carried out in a Hoefer Mighty Small II SE-250 chamber at room temperature. Power supply conditions were 15 mA for each gel and maximum 300 V. After electrophoresis the gels were washed with distilled water and silver stained.

### 2.4.1 Silver Staining

Polyacrylamide gels were silver stained with the Silver Staining Kit (*Amersham Pharmacia Biotec AB, 17-1150-01*). All reagent solutions were freshly prepared before use.

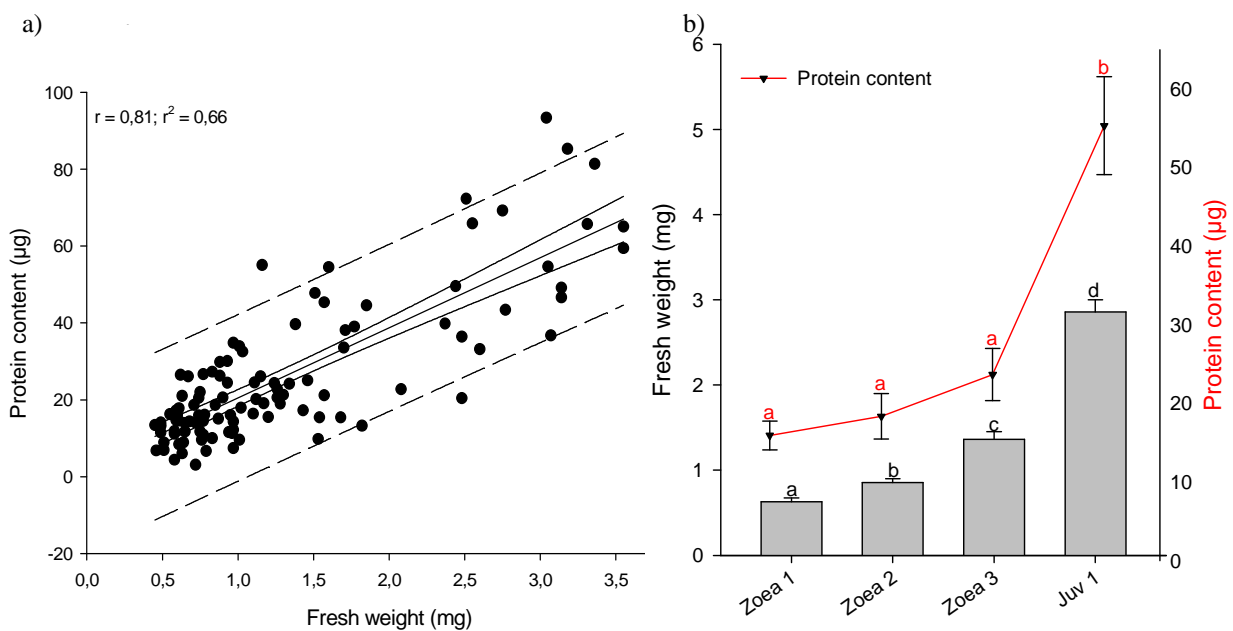
The gels were briefly washed with distilled water and then soaked for 30 min in the fixation solution. Thereafter, the gels were incubated for another 30 min in the sensitizing solution and then washed 3 times for 5 min with distilled water. Then they were incubated in the silver reaction solution for another 20 min and again briefly washed with distilled water. Protein bands became visible after soaking the gels for 2 to 5 min in the developing solution. This reaction was stopped using the stopping solution as soon as dots became visible. A last washing step with distilled water was done (three times for 5 min) and then protein dots were photographed with a gel documentation system (*Biorad, ChemiDoc, program PDQuest*).



### 3. Results

The size, the fresh weight, and the protein content of the claws increased significantly during the development of the lobster larvae. Moreover, the fresh weight and protein content displayed a significant correlation (Fig. 3.1.a;  $r = 0.81$ ;  $r^2 = 0.66$ ,  $p < 0.001$ ).

The weight of a pair of claws increased significantly from stage to stage (Fig. 3.1.b). The claws of the zoea 1 larvae weighed on average 0.63 mg. The weight increased until the first juvenile stage to about 2.2 mg. The protein content rose continuously from 15.95  $\mu\text{g}$  in zoea 1 to 23.68  $\mu\text{g}$  in zoea 3. Between zoea 3 and juv 1 the protein content increased about 2.3-fold.

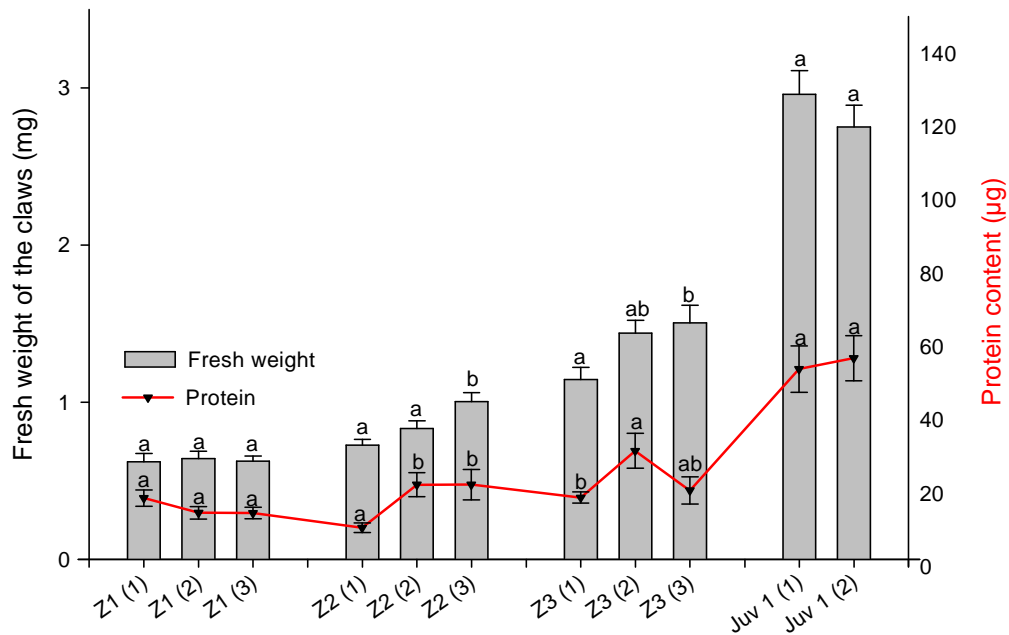


**Fig. 3.1 a) Correlation between the protein contents of the claws and their fresh weights:** Indicated is the regression line with the confidence intervals (95%, dashed line). The solid interval lines indicate the predicted intervals.

**b) Claw fresh weight and protein content per developmental stage:** The bars represent the average fresh weight of a pair of claws. The curve represents the average protein content of a pair of claws. The Values are given as means  $\pm$  SEM (n=10). Different letters indicate significant differences between the groups

The weight and the protein content did not change significantly within the three molt stages of the zoea 1 (Fig. 3.2). Both values, however, increased in zoea 2 larvae where post-molt and pre-molt animals displayed significant differences. This trend continued for the fresh weight within the zoea 3 larvae. The claws of pre-molt zoea 3 individuals were significantly heavier than those of post-molt zoea 3. The protein content rose from the post-molt to the inter-molt individuals significantly but the pre-molt animals possessed less protein again. The two

juvenile molt stages showed significantly higher weights than the larvae but they did not differ in their protein content.

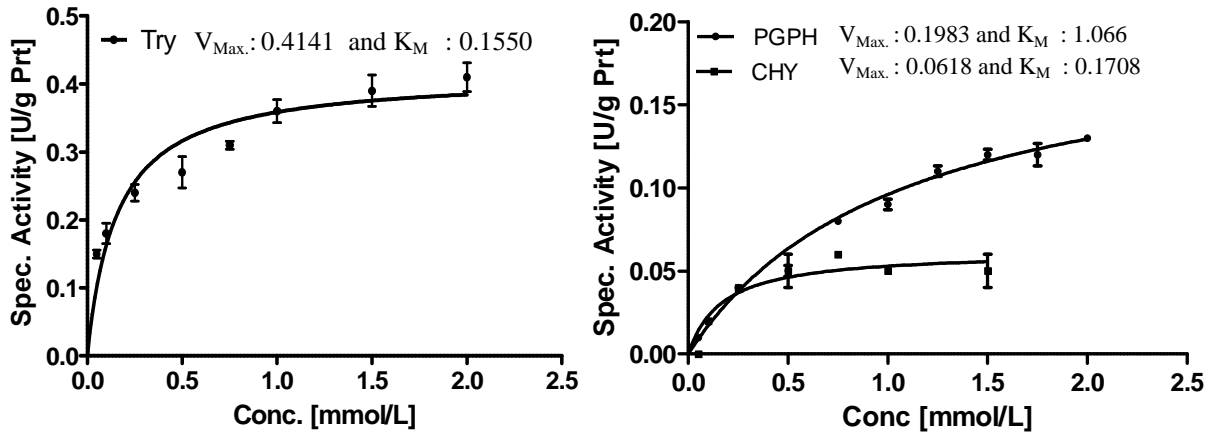


**Fig. 3.2 Claw fresh weight and protein content per molt stage:** Z1 – Z3 = zoea 1 to zoea 3, juv 1 = Juvenil 1, Numbers 1 – 3 in the brackets indicate the molt stage: (1) = freshly hatched or post-molt stages, (2) = inter-molt stages, (3) = pre-molt stages. Values are given as average amounts  $\pm$  SEM, different letters on top of the bars indicate significant differences between the groups.

### 3.1. Enzymatic measurements

#### 3.1.1 Michaelis-Menten Kinetics

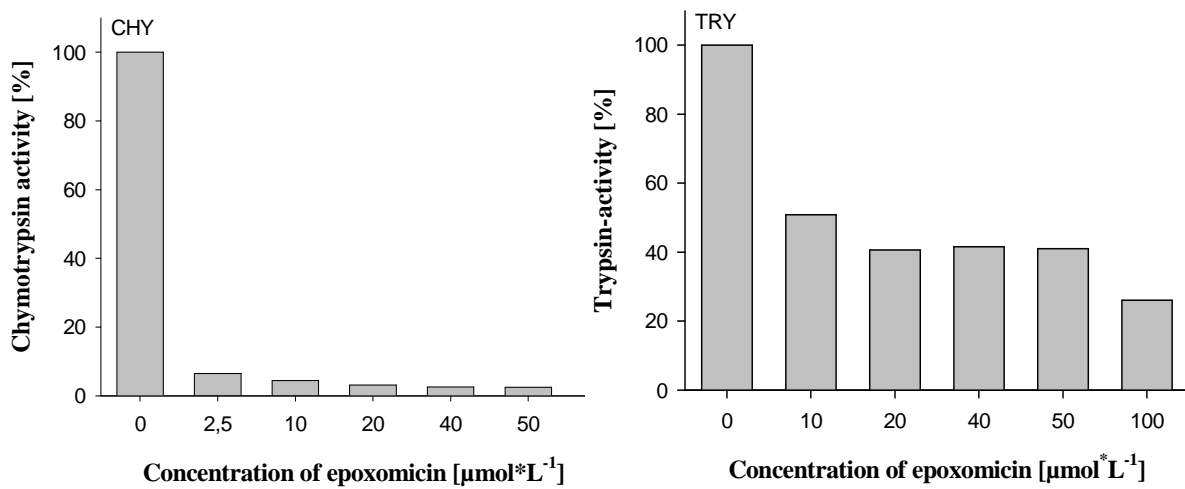
The three proteasomal activities displayed great differences in maximum activity and affinity towards the substrates. The trypsin-like site revealed the highest activity ( $V_{Max} = 0.4141 U \cdot g^{-1}$ ) as well as the highest affinity towards the substrate ( $K_M = 0.1550 \text{ mmol} \cdot L^{-1}$ , Fig. 3.3, on the left). The chymotrypsin-like site possessed the lowest activity ( $V_{Max} = 0.0618$ ) but its affinity towards the substrate ( $K_M = 0.1708$ ) was only slightly lower than the affinity of the trypsin-like activity (Fig. 3.3, on the right). The values obtained for the PGPH-like activity differed most from the results of the other two activities. While the activity was about half of the activity calculated for the trypsin-like site  $V_{Max} = 0.1983$ , the affinity was lowest ( $K_M = 1.066$ ).



**Fig. 3.3 Michaelis-Menten kinetics for the trypsin-like (left), chymotrypsin-like and PGPH-like activities (right) of the lobster proteasome:** The specific activities of the proteasome were assayed at different concentrations of substrates ( $\text{mmol}\cdot\text{L}^{-1}$ ) and expressed as mean  $\pm$ SEM.

### 3.1.2 Inhibition and activation of proteasomal activities

The trypsin-like and chymotrypsin-like proteasomal activities of the claw muscle extract of the adult European lobster were significantly inhibited by epoxomycin (**Fig. 3.4**).



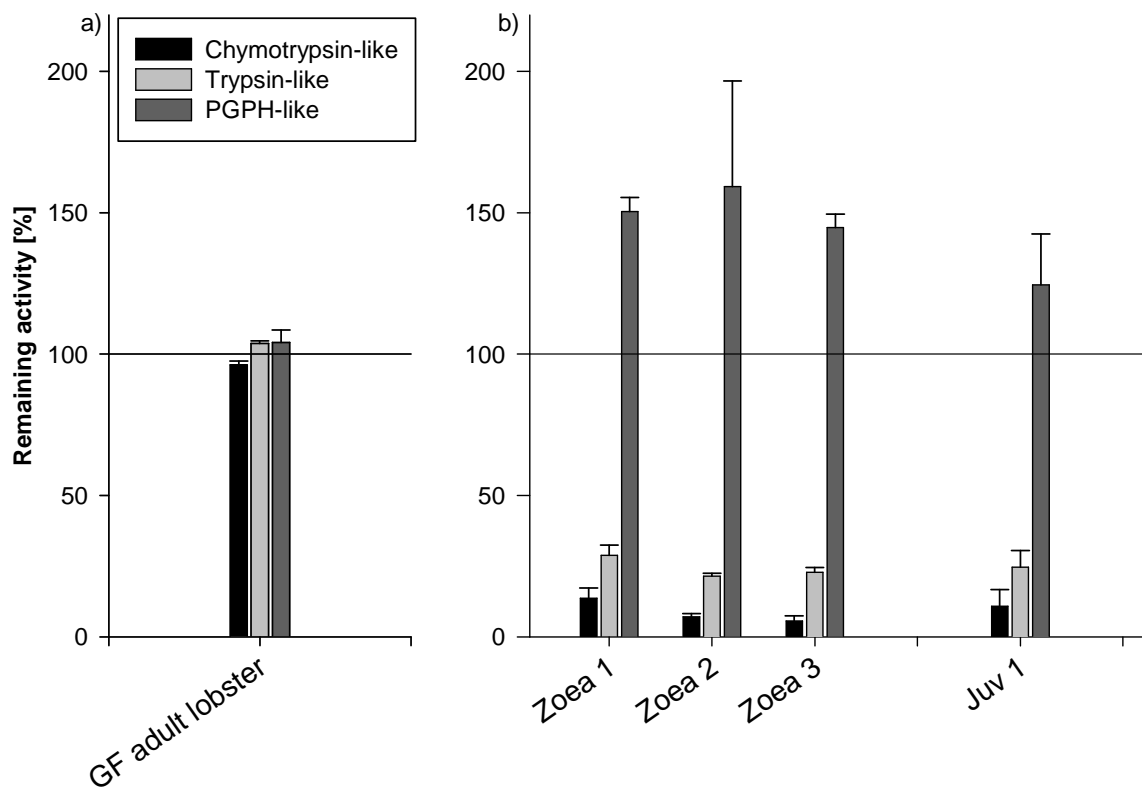
**Fig. 3.4 Inhibition of the proteasomal activity by epoxomycin:** The residual activities (%) are presented for the chymotrypsin-like activity (on the left) and for the trypsin-like activities (on the right) at different concentrations of epoxomycin ( $\mu\text{mol}\cdot\text{L}^{-1}$ ).

Over 93 % of the chymotrypsin-like activity was inhibited when epoxomycin was present even in the lowest concentrations of  $2.5\ \mu\text{mol}\cdot\text{L}^{-1}$ . Around 50 % of the trypsin-like activity was inhibited by  $10\ \mu\text{mol}\cdot\text{L}^{-1}$  epoxomycin. Increasing the concentration of epoxomycin did not considerably enhance the inhibition of the chymotrypsin-like activity. At the highest

concentration of  $50 \mu\text{mol}\cdot\text{L}^{-1}$  only 3 % of the initial activity remained. The trypsin-like activity was inhibited by 75% with the highest epoxomycin concentration of  $100 \mu\text{mol}\cdot\text{L}^{-1}$ .

In the gastric fluids of the adult European lobsters the trypsin-like, the chymotrypsin-like, and the PGPH-like activities were not affected by epoxomycin (Fig. 3.5.a). The chymotrypsin-like activity was only reduced by 3 %. The trypsin-like activity (103 %) and the PGPH-like activity (104 %) were slightly higher than in the control assays.

In contrast to the gastric fluid, the activities of the larval and juvenile lobster samples were highly affected by the inhibitor as well as by the activator (Fig. 3.5.b).



**Fig. 3.5 Effects of epoxomycin and PA28 on the proteasomal activity:**

a) Effect of epoxomycin and PA28 on the digestive enzymes trypsin, chymotrypsin and cathepsin in the gastric fluid of adult European lobsters.

b) Effect of epoxomycin and PA28 onto the three proteasomal activities of the larval and juvenile stages, zoea 1 to zoea 3 and the first juvenile stage. Values are expressed as remaining activity in %. The activity of the untreated sample was set as 100 %

The chymotrypsin-like activities of all investigated stages were inhibited by more than 90 %. The highest inhibition occurred in the third zoea stage where only 5.6 % of the initial activity remained. The average inhibition rate amounted to 90.7 % whereas no significant differences appeared between the stages. The trypsin-like activity was inhibited on average by about 75

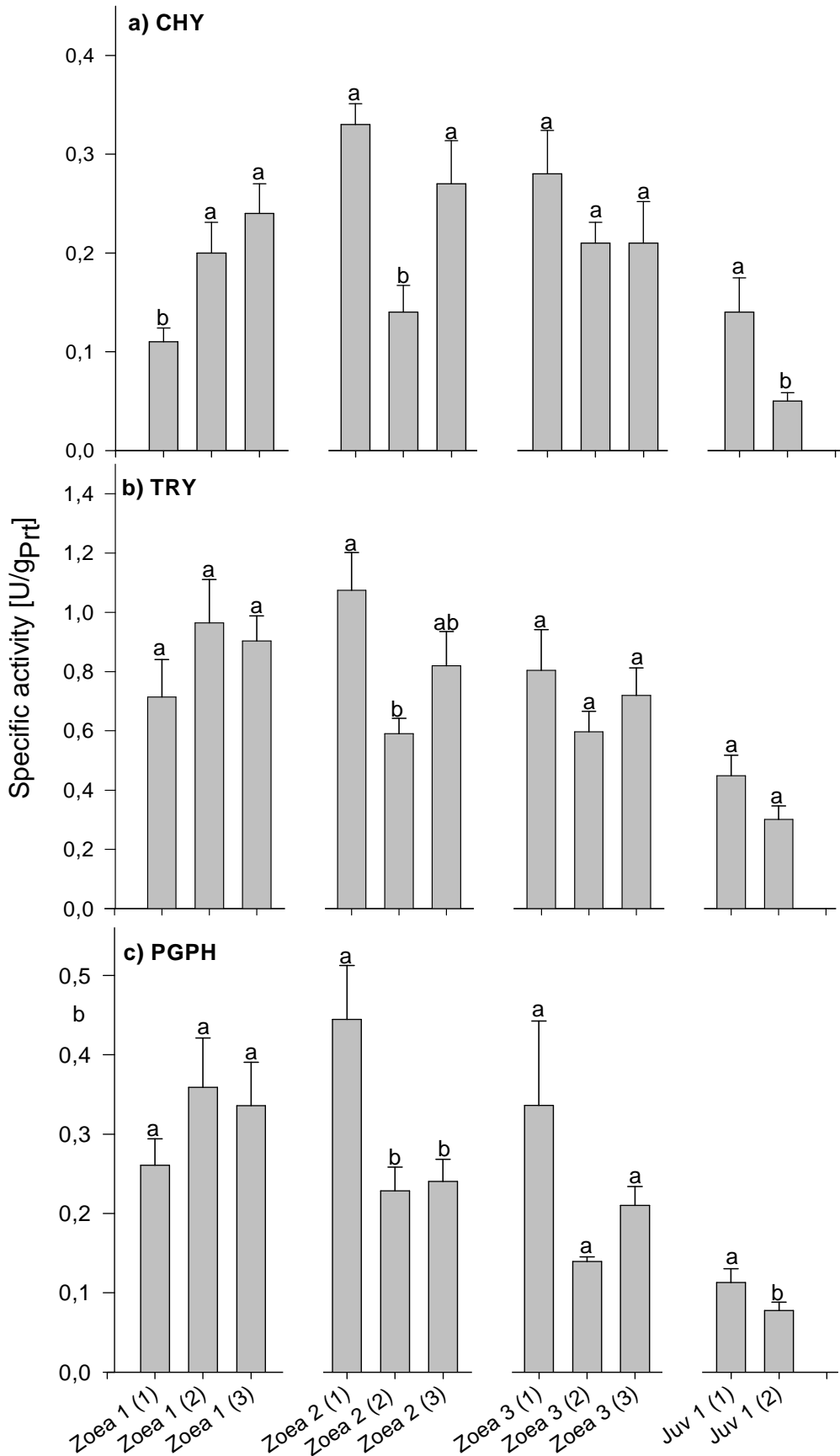
%. Like for the chymotrypsin-like activity no differences between the stages were detected. The endogenous activator complex PA28 enhanced PGPH activity on average by 45 %.

### 3.1.3 Enzymatic activity of the 20S Proteasome

The catalytic activities of the three active sides changed significantly within the larval development. The trypsin-like, the chymotrypsin-like and the PGPH-like activities increased within the zoea 1 and remained at a high level until zoea 3 (results not shown). All three activities decreased significantly when the larvae developed into the juvenile stage.

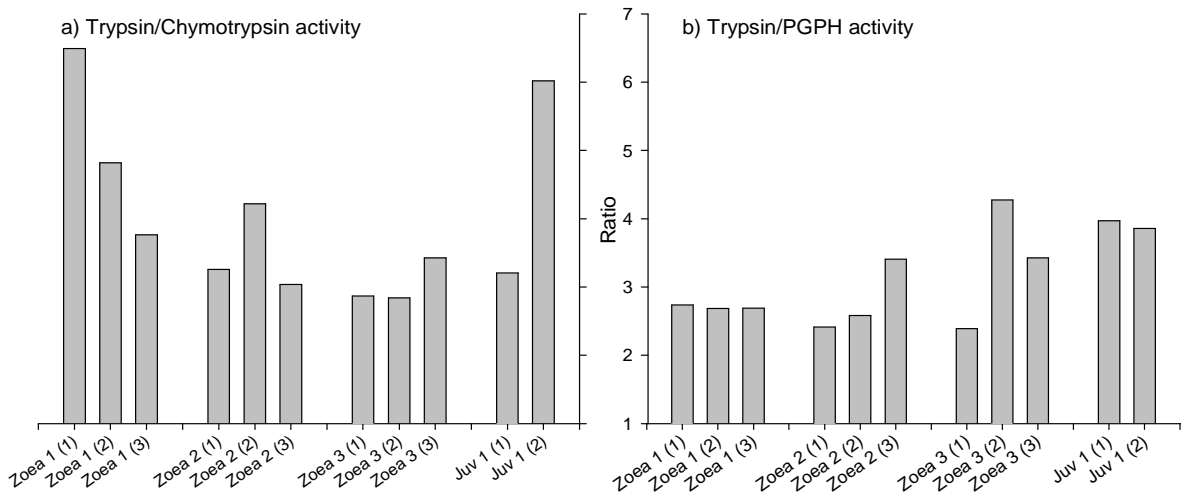
Changes of activities were detected between the different molt stages (Fig. 3.6 a – c). The chymotrypsin-like activity increased within zoea 1 individuals from initially  $0.11 \text{ U} \cdot \text{g}^{-1}$  and reached highest activities in post-molt zoea 2 individuals with  $0.33 \text{ U} \cdot \text{g}^{-1}$  (Fig. 3.6 a). Then, the activity decreased drastically in inter-molt individuals ( $0.14 \text{ U} \cdot \text{g}^{-1}$ ) but reached highest activities again in the subsequent pre-molt individuals. Although, the activity did not change significantly within zoea 3 molt stages, a decreasing trend was evident. This trend continued in the juvenile stage where the activity decreased from  $0.14 \text{ U} \cdot \text{g}^{-1}$  in post-molt individuals to  $0.05 \text{ U} \cdot \text{g}^{-1}$  in inter-molt juvenile lobsters.

Similar trends were also found for the trypsin-like and the PGPH-like activities (Fig. 3.6 b and c). In both cases, the activities showed an increasing, though not significant, trend from freshly hatched zoea 1 larvae to the post-molt individuals of this stage. The inter-molt zoea 2 possessed lowest trypsin-like ( $0.59 \text{ U} \cdot \text{g}^{-1}$ ) and PGPH-like ( $0.23 \text{ U} \cdot \text{g}^{-1}$ ) activities and both activities increased again in post-molt zoea 3 individuals. Both activities decreased in inter-molt individuals of zoea 3. This was followed by an increase in pre-molt individuals again, although these trends were not significant. In the juvenile lobsters both activities decreased again.



**Fig.3.6 Proteasomal activities:** Average chymotrypsin-like activities (a) trypsin-like activities (b) and the PGPH-like activities (c) within the investigated larval and juvenile stages (n=10, mean ± SEM). Different letters indicate significant differences between the groups.

Each stage possessed an individual ratio between the three proteasomal activities (Fig. 3.7 a) and b)). In all stages the trypsin-like activity dominated.



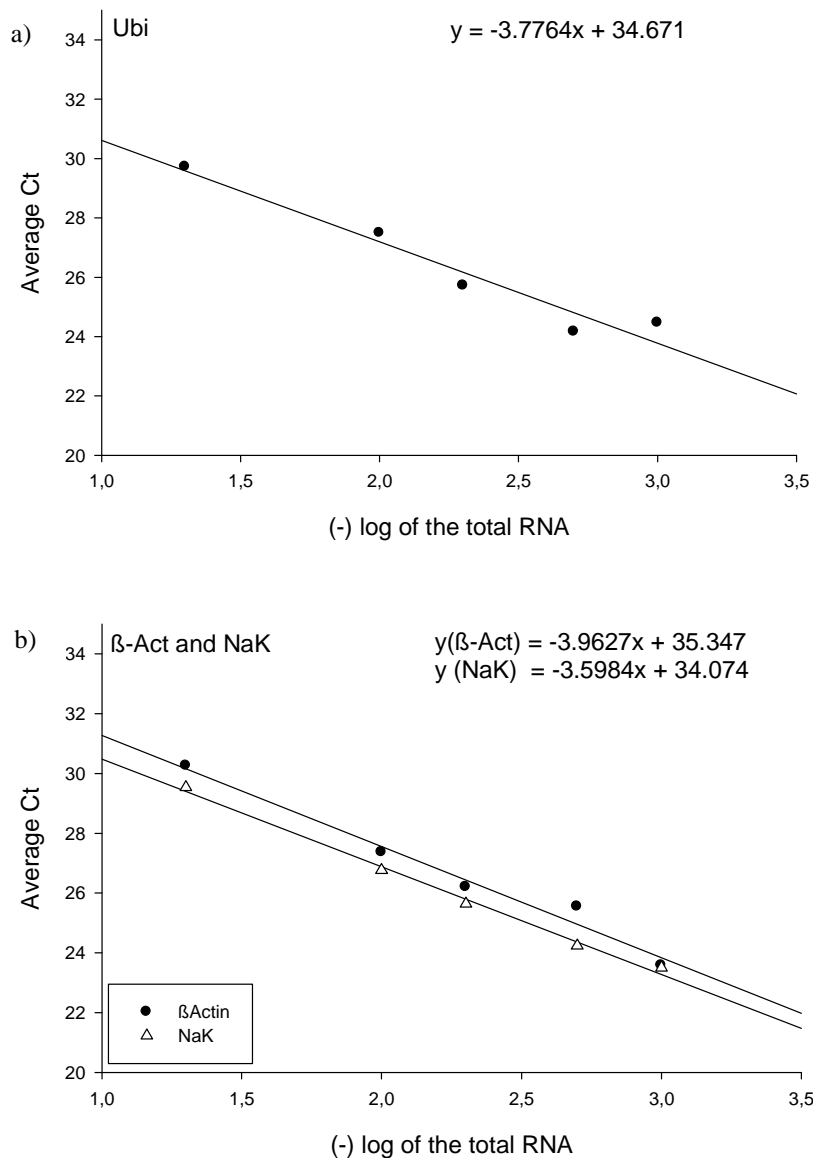
**Fig. 3.7 Ratios between the specific proteasomal activities:** a) Trypsin-like side vs. chymotrypsin-like side b) Trypsin-like side vs. PGPH like side

In freshly hatched zoea 1 individuals, the trypsin-like activity was more than 6 times higher than the chymotrypsin-like activity and almost 3 times higher than the PGPH-like activity. In the subsequent stages, until post-molt zoea 2, the ratio of trypsin to chymotrypsin decreased to about 3. The ratio of trypsin to PGPH did not change within these stages. Inter-molt zoea 2 animals showed again higher trypsin-like activities. The ratio changed in the following stages in favor to the chymotrypsin like activity until the larvae developed into post-molt juvenile lobsters. As soon as the juvenile lobsters passed the molt, the ratio increased until the trypsin-like activity was 6-fold higher than the chymotrypsin-like activity. The ratio of trypsin vs. PGPH did not change until they larvae reached the inter-molt zoea 3 stages. There, the PGPH-activity decreased and the trypsin-like activity was 4 times higher than the PGPH-activity. The ratio between the PGPH and the chymotrypsin activity ranged at around 1 showing that both activities were similarly high. Within the most stages the PHPG-like activity was on average 0.5 times higher than those of the chymotrypsin-like side. However, in the pre-molt zoea 2 and the inter-molt zoea 3 stages the ratio changed in favor to the chymotrypsin-like activity. In these two stages the chymotrypsin-like activity was slightly higher than the PGPH activity (max. 1.5 fold higher).

## 3.2 Quantitative Real-Time PCR

### 3.2.1 Primer and sample evaluation

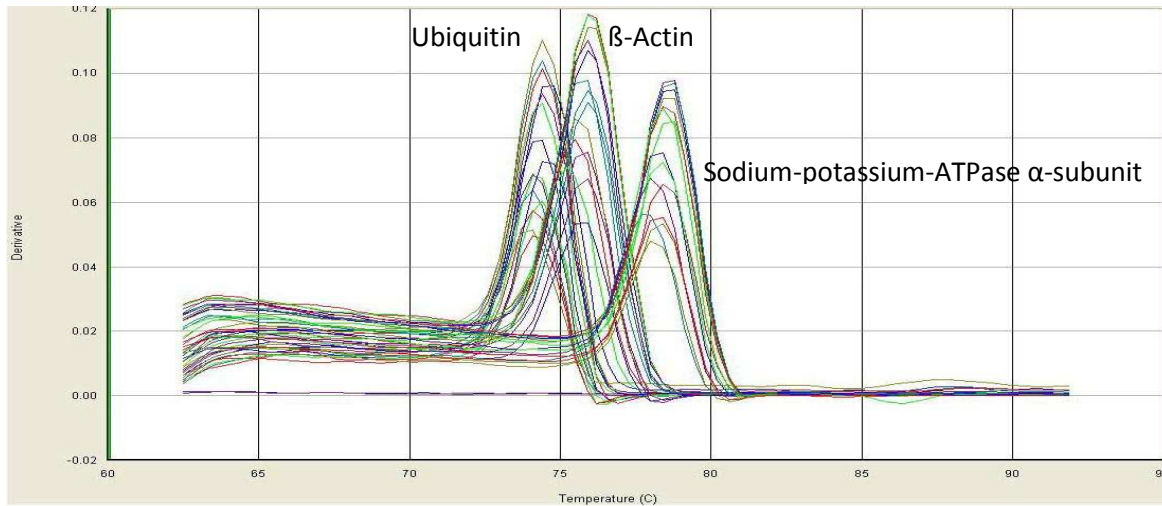
The amplicates for ubiquitin,  $\beta$ -actin and the sodium-potassium ATPase  $\alpha$ -subunit were clearly detected in the dilution series of the cDNA template. Only the  $\alpha$ -Actin gene was not amplified in the PCR and accordingly, the standard curves were only created for the three remaining amplicates (Fig. 3.8).



**Fig. 3.8 a) and b) Standard curves of the ubiquitin, the  $\beta$ -Actin and the sodium-potassium ATPase  $\alpha$ -subunit:** The average Ct-values were plotted against the log of the applied RNA ( $\mu$ g). The linear equation of each regression is given in the right upper corner



The “no template control” and the “no cDNA control” were never detected within the Real-Time PCR for any gene. The dissociation curve analysis showed that only one template was amplified for each gene (Fig. 3.9). The melting points occurred at an average temperature of 74.38 °C for the ubiquitin amplificates, 75.25 °C for the  $\beta$ -actin amplificates, and 78.10 °C for the sodium-potassium ATPase  $\alpha$ -subunit.



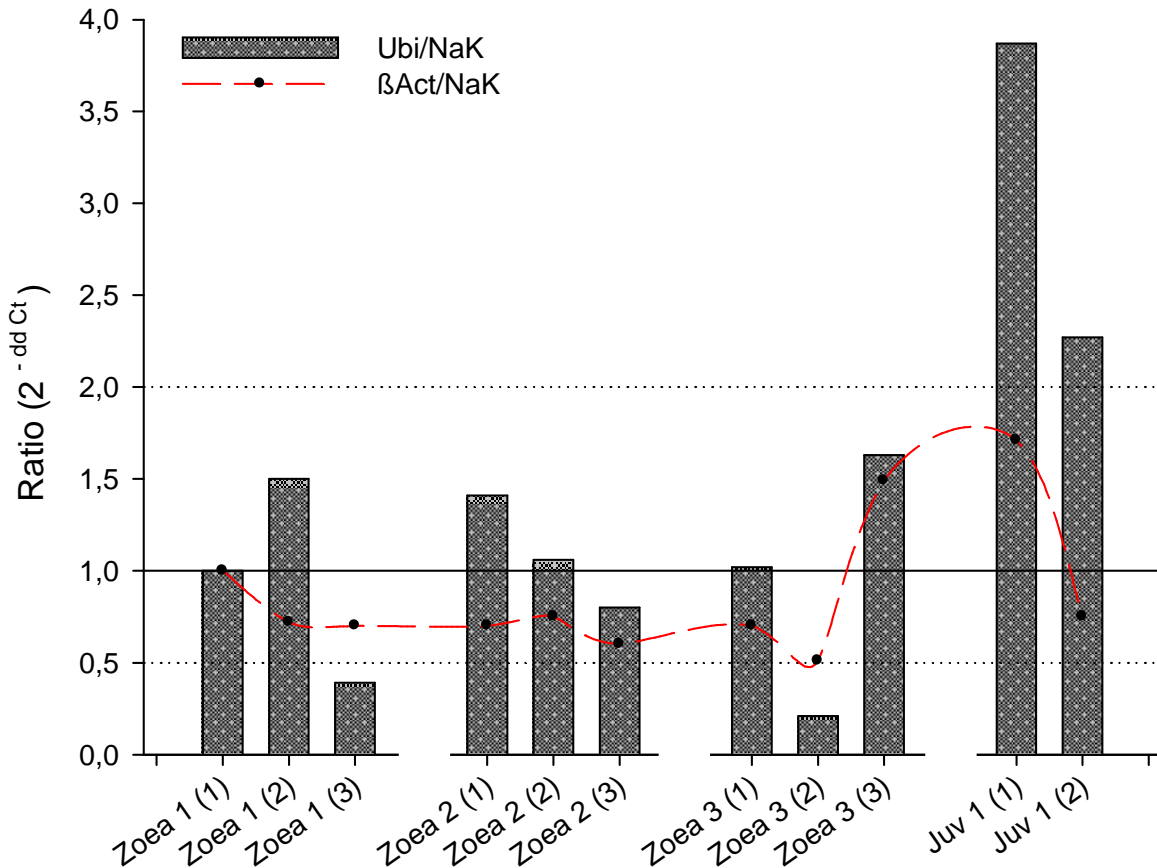
**Fig. 3.9 Dissociation curve analysis of the PCR products:** Shown are the melting curves of the ubiquitin,  $\beta$ -Actin and sodium-potassium ATPase  $\alpha$ -subunit amplificates.

The mRNA quantities of the sodium-potassium ATPase  $\alpha$ -subunit changed less within the different developmental stages than the quantities of the  $\beta$ -actin (Data not shown). Therefore, the sodium-potassium ATPase  $\alpha$ -subunit was chosen as a housekeeping gene. Hence, beside the ubiquitin gene expression, the  $\beta$ -actin expression could be investigated as well.

The developmental stages displayed slight variations in the ubiquitin and the  $\beta$ -actin mRNA quantities (Fig. 3.10). These variations between the stages were different for both investigated amplificates. Two-fold changes, i.e. the duplication or the halving of the relative quantities, were used as references for predicting significant differences.

The quantities of the ubiquitin mRNA levels differed less than 2-fold within the first two larval stages zoea 1 and zoea 2. The only exception was seen for the pre-molt zoea 1 where the quantity of mRNA decreased about 3-fold in comparison to the inter-molt zoea 1. The mRNA quantity decreased from post-molt zoea 3 to inter-molt zoea 3 about 4-fold and immediately increased in the pre-molt zoea 3 about nearly 6-fold. The greatest change in comparison to the freshly hatched zoea 1 larvae was reached in the post-molt juvenile lobster;

there the quantities change about 8-fold. The quantity of ubiquitin mRNA decreased again about 3.6-fold in inter-molt juveniles.



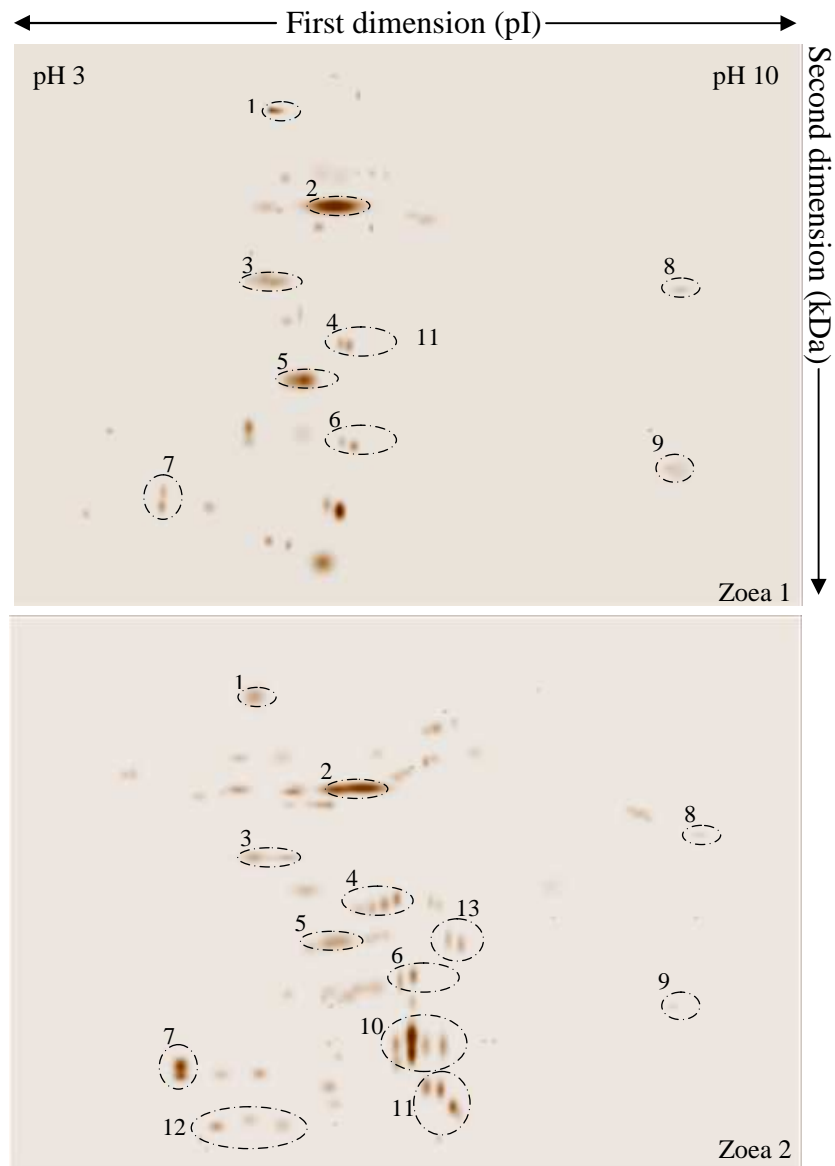
**Fig.3.10** Quantities of the ubiquitin and β-actin mRNA levels in different molt stages of the European lobster larvae. The levels of mRNA are expressed as the relative x-fold change “Ratio (2<sup>-ΔΔCt</sup>)” in comparison to the control group, the freshly hatched zoea 1. The level of this group was set to be 1, indicated by the black horizontal line. Doubled or halved quantities are indicated by dotted lines (ratios 0.5 and 2).The bars symbolize the quantity of ubiquitin, whereas the red line symbolizes the level of the β-actin mRNA.

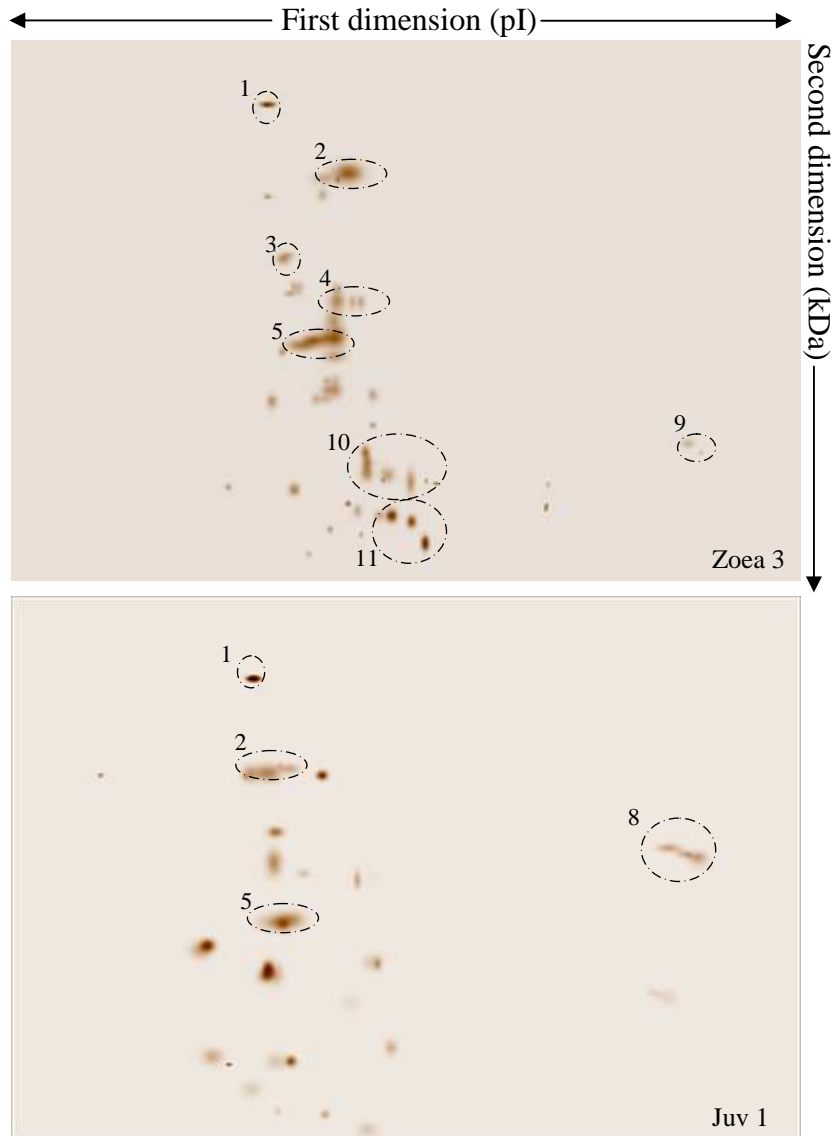
The β-actin quantity remained up to the inter-molt zoea 3 at a level of 0.7-fold. Towards the pre-molt zoea 3, the β-actin quantity increased about 3-fold and reached the highest value in the post-molt juvenile stage with a 1.7-fold change of quantity compared those of the freshly hatched zoea 1. In the inter-molt juvenile lobster sample the level decreased again to the quantities observed in the first juvenile stages.

### 3.3. 2D-PAGE

The claw extracts of the four inter-molt stages displayed a great variety within their detectable protein spots (Fig 3.11a, the major protein spots were labeled with numbers). In all stages the protein dots were mainly detected at acidic to slightly neutral pI-values. Except two proteins with high, basic pI values, hardly any proteins were found in a neutral to basic range. Identical spots within the stages differed in their intensity (e.g. 1, 2, 5).

The zoea 1 larva and the first juvenile had mainly proteins with a slightly more acidic pI than the the second and third zoea stages. Furthermore, in the samples of the zoea 2 and zoea 3 larvae more proteins were detected in general with a lower molecular weight (e.g. protein pattern labeled with 10, 11, 12, 13).





**Fig.3.11 Two Dimensional gel images of the 4 inter-molt stages (zoea 1 to juv 1):** From the left to the right side the pH gradient proceed, labeled with acidic (pH 3) and basic (pH10). The numbered circles indicate the same protein spots within different samples.

Although the proteins of single larvae claws could be successfully separated by 2D-electrophoresis, the immunochemical detection of the subunits failed. It remains to be investigated whether the amount of protein was too low or the cross-reactivity of the antibodies with the lobster proteasome subunits was too poor. Since there is no specific product on the market, the lobster proteasome has to be isolated and antibodies have to be raised against the lobster subunits. This task, however, is far beyond the aim of the present bachelor thesis but, hopefully, will be considered in future research activities.

## 4. Discussion

The presented results indicate that the 20S proteasome plays an important role already during the larval development of the European lobster. The trypsin-like, the chymotrypsin-like and the PGPH-like sites showed high catalytic activities within all larval stages. In the first juvenile stage the activities decreased remarkably. In comparison with the claw muscle tissue of adult European lobsters the proteasomal activities of the larvae seem to be several times higher than in adults. Accordingly, it is very likely that these high activities are indicative for the extensive physiological and anatomical changes the larvae go through during their development ([Charmantier 1991](#)).

### 4.1 Variations of proteasomal activity levels

The activities varied distinctly between stages and within stages. While within zoea 1 larvae the activities continuously increased, the activity patterns in zoea 2 and zoea 3 seemed to be more related to the molt stage. It is most likely that in the first larval stage general anabolic processes occur. The continuously rising activities may reflect the continuous formation of the proteasome. Lower activities in the inter-molt individuals of zoea 2 and zoea 3 may reflect a period of lower protein turnover and, therefore, of lower proteasomal activities due to a down-regulation of the enzyme. In contrast, the increasing activities particularly of the trypsin-like and the chymotrypsin-like sites of the pre-molt zoea 2 may indicate a phase of enhanced protein turnover closely before molt. The increase of activities was most distinct in the pre-molt zoea 2. In zoea 3 pre-molt individuals the activities followed a similar trend but the increase of activities in the pre-molt stage was not statistically significant.

The lacking increase of activities in pre-molt zoea 3 may be explained by the difficult determination of the individuals during sampling. The only possibility to identify pre-molt larvae was to take other larvae as a clue. Larvae of the first two zoea stages pass their development on average within 4 and 8 days, respectively. Furthermore, larvae of these stages molted into the next stage almost simultaneously which made it relatively easy to collect them at a defined time shortly before molt. The greater variation shown for the duration of the zoea 2 stage (see Material and Methods, [Tab.2.1](#)) is due to different hatches of larvae, which still molted simultaneously but needed slightly different periods for their development. In contrast, zoea 3 larvae needed almost 13 days to reach the next stage and, moreover, they did not molt as simultaneously as the earlier stages did. For example, if the first larva of a hatch molted

after 11 days then the other larvae, which were sampled for analysis at this time, may still have needed another 2-3 days which, however, is quite far before molt. Some few larvae even did not molt into the juvenile stage at all. They remained in the zoea 3 stage and died about one week after their predicted molt date. Thus, these potentially falsely classified individuals might have caused a great scatter in the proteasomal activity data and, apparently, may have lead to an underestimation of the enzyme activity values. A second feasible reason for the lack of activity increase may be related to the transition from larval metabolic processes to juvenile metabolic processes. The first juvenile stage showed significantly lower proteasomal activities than the zoea 2 and zoea 3 stages. Since the larval development of the lobster is more continuous than in other decapods lacking a distinct metamorphosis from e.g. the megalopa into the first juvenile stage it might be speculated that the late zoea 3 already start to adopt the metabolic characteristics of the juvenile. Accordingly, the high proteasomal activities and the molt-related activity patterns of the larvae may be masked by the generally lower proteasomal activities of the upcoming juvenile stage.

#### **4.2 Variation of proteasomal activity sites**













Similar to the enzymatic properties of the latent 20S proteasome which was isolated from adult lobster muscle tissue ([Mykles 1997](#)), the proteasome of the larvae possessed highest trypsin-like activities and remarkably lower chymotrypsin-like and PGPH-like activities. Remarkably, the ratios between the proteasomal activities changed during the larval development. While in freshly hatched larvae and in juvenile inter-molt individuals the trypsin-like site was dominant, the chymotrypsin-like site seems to gain relevance in the Zoea 2 and Zoea 3 stages.

The proteasome is characterized by high plasticity and, moreover, distinct tissue allocation ([Cardozo et al. 1995](#), [Mykles 1999b](#), [Dahlmann 2000](#)). Several authors studied the proteasome in embryonic tissues ([Haas and Kloetzel 1989](#), [Klein et al. 1990](#)) and [Young et al. \(1991\)](#) described a change in the enzymatic activities during the embryonic development of chick skeletal muscle. Most considerable in this study was the fact that there is a short period within the development of the chick skeletal muscle in which the chymotrypsin-like activity increased while the other activities remained at the same level. Moreover, the proteasomal activities decrease in the further progress of development. These results are in good agreement with my own observations which showed that within the larval stages the

chymotrypsin-like activity of the 20S proteasome increases but decreases again in the juvenile stages.

All catalytic sites are closely interlinked and influence each other through allosteric interactions for most efficient degradation (Kisselev et al. 1999). For example it was shown that the two chymotrypsin-like sites cooperatively influence each other. Moreover, substrate binding to the chymotrypsin-like site stimulates the activity of the PGPH-like site. Therefore, Kisselev et al. (1999) suggested a “bite-chew” mechanism to describe the proteasomal degradation of proteins (Tab.4.1). The polypeptides are initially bound by one of the two chymotrypsin-like sites and cleft into fragments. Simultaneously, the other 4 catalytic sites are stimulated as they take over the further degradation. With increasing trypsin-like and PGPH-like activities, the chymotrypsin-like site is progressively inhibited to prevent excessive generation of fragments. When the fragments are completely degraded and released, the chymotrypsin-like site is again activated. This mechanism implies that the chymotrypsin-like sites of the 20S proteasome are those which control the rate of protein degradation (Heinemeyer et al. 1997, Arendt et al. 1997, Kisselev et al. 1999).

**Tab. 4.1 “Bite-and-Chewing” mechanism of the 20S proteasome for protein degradation** (after Kisselev 1999): The functional states of the active sites are illustrated in green (active state), yellow (reduced activity) or red (inhibited activity)

Site	Functional State of the active sites			
	Beginning	“Bite”	“Chewing”	End
		of a incoming polypeptide	of a incoming polypeptide	
CHY				
TRY				
PGPH				

The controlling functions of the chymotrypsin-like sites were supported by studies which showed that the inhibition or inactivation of these sites through mutations or site specific inhibitor significantly influenced the proteasomal activity and the cell survival. The deactivation of the two other active sites seemed to have only a weak influence (Lee and Goldberg 1996, Heinemeyer et al. 1997). However, more recent investigations revised this assumption, because (i.g. Kisselev et al. 2006) proved that a significant decrease in the whole protein breakdown (*in vitro and in vivo*) could not be achieved by inhibition of the chymotrypsin-like sites alone. Only the inhibition of the chymotrypsin-like sites and the

simultaneous inhibition of either the trypsin-like site or the PGPH-like site lead to a significant decrease of activity. Together with the “bite-chew” mechanism, these findings imply that although the chymotrypsin-like sites seem to limit the protein breakdown rate, all three active sites are closely interlinked and, only together, they perform the effective degradation of proteins. The mechanism proposed by [Kisselev \(1999\)](#) strongly indicates that the shift from a very dominant to a less dominant trypsin-like site, and instead rising chymotrypsin-like activities, may be another evidence for a better adjustment of the proteasome to facilitate higher protein turnover rates.

The lobster larvae showed a distinct and continuous change from lower chymotrypsin-like activity in the first stage to higher chymotrypsin-like activities during the entire larval development. Only in the juveniles the chymotrypsin-like activity was reduced again and both enzymes reached the same ratio as in the first larval stage. These results indicate that, beside the general increase of the proteolytic enzyme activities, the proteasome is adjusted to better cope with higher protein turnover rates during the entire larval development

#### **4.3 Proteasome and muscle claw atrophy**

The proteasomal involvement of muscle claw atrophy in adult decapod crustaceans was mainly studied by [D.L. Mykles](#) and colleagues. [Mykles \(1991\)](#) proposed a direct involvement of the ATP/Ubiquitin-dependent proteolytic pathway of the 26S proteasome in the claw muscle atrophy of adult decapod crustaceans. [Mykles \(1992, 1999a, 1999b\)](#) showed that the proteasome is able to degrade myofibrillar proteins, at least *in vitro*. However, myofibrillar proteins turned out to be only poor substrates for the basal conformation of the multicatalytic complex (20S proteasome, [Mykles and Haire, 1991](#)). Only the heat-activated proteasomal conformation, mainly the branched-chain-amino-acid-preferring activity (BRAAP), was able to degrade myofibrillar proteins such as myosin, troponin or tropomyosin at a remarkable extent. Other studies figured out that the BRAAP activity is not due to an independent additional active site, but this activity is actually performed by the chymotrypsin and the PGPH-like sites ([McCormack, 1998](#)). This result again underlines the suggestion that the chymotrypsin-like sites seem to have a specific relevance for the degradation of myofibrillar proteins.



#### 4.4 Proteasome and tissue-reorganization

I showed in this study that the latent, not purified 20S proteasome exhibits a variety of catalytic activities within the larval development which seems to reflect the physiological state of the 20S proteasome *in vivo*. However, it is not clear whether the changes in the catalytic preferences as well as the increasing capabilities of degradation are solely due to myofibrillar degradation or also participate in other cellular processes. The anatomical design of the claws implies that already the larvae need to reduce and reorganize muscle tissue before ecdysis. So it seems obligatory that the proteasome might also be involved in these reorganization processes.

The enormous reduction of claw muscle tissue prior to each molt confront adult crustaceans with serious degradation- and remodeling processes. Although, the muscle mass has to be reduced so far that the tissue fits through the narrow joint, the muscle has to maintain its contractile function. This is done through a distinct remodeling of the myofibrillar structures (Mykles and Skinner 1981, Ismail and Mykles 1992). Surprisingly, the protein synthesis in the claw tissues is not reduced in pre-molt crustaceans, but instead significantly increased (Dahlmann et al. 1995, El Haj et al. 1996, Mykles 1997). The reason for the steady protein synthesis is a complex mechanism to re-modulate the myofibrillar structures and to reduce the muscle tissue while the contractile function should not be impaired (Mykles and Skinner 1981). I assume that this mechanism, as it represents a period of enhanced protein turnover, will strongly depend on proteasomal activity as well. The proteasome is one of the main regulators of the cell-cycle (Richter-Ruoff and Wolf 1993, Naujokat and Hoffmann 2002), differentiation processes such as remodeling (De Diego et al. 2001), and growth. Therefore it seems likely that especially in developmental stages which are characterized by high mitotic activity, growth and high transcription rates might demand an active and regulating proteasome. Unfortunately, only a few studies have been done so far which investigated the activities of the proteasome within developmental stages and a comparison with lobster molt processes is somehow inaccurate. Klein and Kloetzel (1990) reported a cell specific accumulation of *Drosophila* proteasome during the early development and its occurrence in proliferating cells. De Diego et al. (2001) assumed that the enzyme plays an important role during the remodeling phase of *Trypanosoma cruzi*. The larvae of clawed lobster seem to be suitable models to study tissue related processes with biochemical as well as histochemical methods. The larvae have short and defined molt phases and they are large enough to provide sufficient material for analyses.

#### 4.5 Expression of ubiquitin during the molt cycle

The quantitative Real-Time PCR indicates that the ubiquitin expression does not show molt depending variations within the first two larval stages. The molt cycle-dependent ubiquitin expression in adult lobster claw muscle tissue observed by various scientists (Shean et al. 1995, Koenders et al. 2002, Spees et al. 2003), seem to start in the third larval stage. Only in this stage, the high, several-fold change of the mRNA quantities indicates that these changes are not due to random variations but to different requirements in protein degradation before the molt. The observation that the greatest change happened in the post-molt juvenile individuals fits quite nicely into this picture. These animals were sampled only a short time after they molt and therefore, the expression of ubiquitin was probably not down-regulated yet. The study of Spees et al. (2003) supports this suggestion as he showed that the poly-ubiquitin mRNA amount reached highest values in animals at their day of molt. In contrast to the ubiquitin, the  $\beta$ -actin quantities did not change that drastically. Within all stages, the  $\beta$ -actin reached never half or double quantities in comparison to the freshly hatched zoea 1.

Unfortunately the qRT-PCR could only be done once though with 10 pooled animals in each assay. The reason for pooling samples was mainly the little size of the larvae. It was not possible to extract enough total RNA (0.4  $\mu$ g) from the claw tissue of one individual. As a consequence, a minimum of ten individuals had to be pooled and therefore not enough larvae were available to run replicates in qRT-PCR. However, it has to be kept in mind, that the received quantities represent the mean amount of ubiquitin or  $\beta$ -actin of 10 individuals per stage. Furthermore, these 10 individuals were analyzed in triplicates, so it is likely that the observed quantities may well represent the approximate level of mRNA of both “genes”. Due to the lack of statistic, the quantity changes cannot be stated as significant but according to Dr. Lucassen (personal pers. com.) a 4-fold changes between two stages most likely would turn out to be significant.

The two target amplicates showed different trends of quantity changes during the larval development. This indicates that the ubiquitin changes are likely due to an ubiquitin specific gene expression.

#### 4.6 Subunit identification

The 2D-gelelectrophoresis was successfully done with the claws for the four inter-molt individuals. The protein spots showed a high variability within each stage. Since proteasomal subunits have a molecular weight between 21 kDa and 32 kDa (Review: [Baumeister et al. 1998](#)) the protein spots of interest will most likely be those found in the lower half of the 2D-Gel.

The protein pattern in zoea 1 fitted better to those pattern of the first juvenile individual than to the pattern of the other two zoea stages. Zoea 2 and zoea 3 again showed higher similarities to each other. These results may indicate that the proteasomal subunit composition does somehow change within the lobster development. The zoea 2 and zoea 3 seem to have an exceptional subunit composition which maybe is due to different metabolic requirements. But to make secured statements about the change in proteasomal subunit composition and the resulting consequences for the proteasomal activities, it would be necessary to identify the subunits and to exactly determine the pI and the molecular weight of each subunit.

Unfortunately, the identification of proteasomal subunits by a specific antibody was not successful (results not shown). Although the antibody was reported to possess cross-reactivity to the lobster proteasome ([Haire et al. 1995](#)), it was not possible to detect any subunits of the 20S proteasome. It is not clear whether the antibody had poor cross-reactivity or the proteasome content of the samples was too low for detection. More comprehensive studies are required to isolate the lobster proteasome and to raise specific antibodies against the subunits.

#### 4.7 Conclusion

The detected strong increase and the courses of proteasomal activities, the variation of activity patterns, and the ubiquitin mRNA expression, clearly indicate an important role of the proteasome in the developmental stages of lobster larvae. The enzyme is accurately adjusted to the different requirements of the claw muscle tissues, depending most likely on the molt stage of the individuals. This is especially evident in pre-molt individuals of the Zoea 2 (and Zoea 3) implying that already the early stages show an molt-induced claw muscle atrophy or myofibrillar reorganization. Furthermore, it seems that especially the second and third larval stages have to cope with cellular processes in which a high proteasome attendance is required. The exceptional activity patterns in inter-molt individuals of zoea 2 and zoea 3 most likely display variations in the subunit pattern of the 20S proteasome. It is the shift from lower

chymotrypsin-like activities in zoea 1 to higher activities in zoea 2 and zoea 3 and finally the shift back to low activities in the juvenile stages which indicate an adjustment of the proteasome to cope with specific physiological tasks during the larval development .

#### **4.8 Perspectives**

I was able demonstrate for the first time the involvement of the 20S proteasome during the larval claw muscle development of the European lobster, *H.gammarus*. But still some of the question I raised at the beginning of this study could not fully be answered. A main future focus would be the identification of proteasomal subunits. Since the antibody apparently did not bind to the lobster proteasome subunits, it appears necessary to purify the 20S proteasome of the European lobster and to raise own and highly specific antibodies. Simultaneously, the expression of subunits within the larval stages has to be quantified by Real-Time PCR approaches. Furthermore, it has to be investigated how the proteasome is regulated and whether this regulation is related to the endocrine processes of the molt cycle. Finally, expression studies of proteasomal activator complexes and maybe PA700 complexes would help to understand the higher activity of the 20S proteasome during the second and third zoea stage.

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## Appendix

### Buffer composition

#### Homogenizing and assay buffer

---

**Homogenizing buffer (after Dawson, 1995)**

---

0.02 M Tris(hydroxymethyl)aminomethane (Tris)

0.001 M Ethylenediaminetetraacetic acid (EDTA)

0.005 M Magnesiumchloride (MgCl<sub>2</sub>)

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pH 7.5

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**Assay buffer (after Dawson, 1995)**

---

0.1 M Tris

---

pH 8.0

#### Discontinuous Western Blotting

---

**Cathode buffer**

---

0.05 M Sodium tetraborate (Borax)

0.10 % SDS

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**Anode buffer**

---

0.05 M Sodium tetraborate (Borax)

15,0 % Methanol

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**Coomassie Brilliant Blue Staining solution**

---

0.05 % (w/v) Coomassie Brilliant Blue G250

10.0 % Acetic Acid

40.0 % Methanol

---

Filled up to 1L with distilled water

---

**Destaining solution**

---

10.0 % Acetic Acid

40.0 % Methanol

---

Filled up to 1L with distilled water



## Two-Dimensional Gel Electrophoresis

### Urea stock solution (8M Urea)

---

25 g urea solved in 50 ml distilled water

---

0.5 g Serdolit MB-1

### Rehydration solution

---

2 % 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)

0.015 M Dithiothreitol (DTT)

0.2 % Ampholytes

dissolved in urea stock solution (8M Urea)

---

Prepared freshly before each use

### Equilibration buffer (stock solution)

---

6 M Urea

30 % Glycerol

2 % Sodium dodecyl sulfate (SDS) solved 0.05 M Tris/HCL (pH 8.8)

---

Storable at room temperature up to two weeks

### Buffer A (per IPG strip)

---

0.05 g DTT

dissolved in 5 ml equilibration stock solution

---

### Buffer B (per IPG strip)

---

0.2 g Iodoacetamide

dissolved in 5 ml equilibration stock solution

---

## Silver Staining

### Fixation solution

---

100.00 ml Ethanol

25.00 ml Acetic Acid

---

Fill up to 250 ml with distilled water

### Sensitizing solution

---

75.00 ml Ethanol

1.25 ml Glutardialdehyde (25 % w/v) – add shortly before use

10.00 ml Sodium thiosulfate (5% w/v)

17.00 g Sodium acetate (one packet)

---

Fill up to 250 ml with distilled water

**Silver reaction solution**

---

25.00 ml Silver nitrate solution (2.5 % w/v)

0.10 ml Formaldehyde (37 % w/v)

---

Fill up to 250 ml with distilled water

**Developing solution**

---

6.25 g Sodium carbonate (one packet)

0.05 ml Formaldehyde (37 % w/v)

---

Fill up to 250 ml with distilled water

**Stopping solution**

---

3.65 g Silver nitrate solution (2.5 % w/v)

---

Fill up to 250 ml with distilled water



