The regulation of digestive enzyme release in the two-spotted field cricket *Gryllus bimaculatus* (de Geer): effects of endogenous and environmental factors

Kumulative Dissertation

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Abbreviations

AST	allatostatin
AST-A	allatostatin type A
AT	acclimation temperature
Ba*p*Na	N_{α}-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride
CA	corpora allata
cDNA	complementary desoxyribonucleic acid
CMC	carboxymethyl cellulose
CMCh	carboxymethyl-chitin-RBV 5R
DNS	dinitrosalicylic acid
dsRNA	double-stranded ribonucleic acid
EC	enzyme commission
EPI	endogenous protease inhibitor
Fig.	figure
G. bimaculatus	*Gryllus bimaculatus*
GHF	glycosyl hydrolase family
GlcNAc	N-acetyl-D-glucosamine
IPM	integrated pest management
IT	incubation temperature
JH	juvenile hormone
K_m	Michaelis-Menten constant
L*p*Na	L-leucine *p*-nitroanilide hydrochloride
Ma	maltose
mRNA	messenger RNA
PG	peritrophic gel
PI	protease inhibitor
PM	peritrophic membrane
PMx	peritrophic matrix
*p*NA	*p*-nitroaniline
*p*NP	*p*-nitrophenol
*p*NPP	*p*-nitrophenyl palmitate
RBV	remazol brilliant violet
RFU	relative fluorescent units
RNA	ribonucleic acid
SBTI	soybean trypsin inhibitor
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SK	sulfakinin
V_{max}	maximum reaction rate

Part I

Synopsis

1 Introduction

Insects represent the most diverse and abundant group of animals on earth, and they have a collectively huge ecological and economic impact. A lot of insect species are vectors of several diseases or are classified as pest insects, causing enormous damage in agriculture, food storage or the building industry (Terra et al., 1996). Therefore, the expansion of our knowledge on their way of living and physiology is more than ever indispensable. The physiology of insect digestion has been investigated intensively over the last decades and a lot of different strategies have been developed to deal with different kinds of pests creating a new area of research (integrated pest management, IPM). Various strategies using plant inhibitors, insect growth regulators, transgenic plants and other ecological friendly pesticides have been tested to control the development and reproduction of pest insects (Digali, 2010).

In spite of the intensive research there are still a lot of questions about the fast adaptation of insects to environmental changes, and the physiology and evolution of the mechanisms of adaptation. To develop new methods of pest control the investigation of these mechanisms as well as our knowledge on the physiology of insect digestion (including the regulation of enzyme release) has to be expanded. Therefore, the present thesis deals with different factors regulating the secretion and activity of proteases (trypsin, aminopeptidase), carbohydrases (α-amylase, cellulase, chitinase) and lipases in the digestive tract of an omnivorous insect, the two spotted field cricket *Gryllus bimaculatus*.

1.1 The morphology of the digestive tract

The insect gut is divided in three regions: foregut (pharynx, oesophagus, crop, proventriculus), midgut (ventriculus, caeca) and hindgut (pylorus, ileum, colon, rectum). The fore- and hindgut are ectodermal derivatives lined with cuticle and, therefore, undergo a moulting process, but the midgut is of entodermal origin without a cuticular lining and often contains a peritrophic matrix (PMx).

G. bimaculatus shows a typical Orthopteroid foregut (Terra, 1990; Chapman, 1998) (Figure 1) containing a large expandable crop and a muscular proventriculus with grinding teeth. After food uptake the crop acts mainly as a storage organ for incorporated food, but in *G. bimaculatus* there is also a first step of protein and carbohydrate digestion by a flow of digestive enzymes from the caeca to the crop (Woodring et al., 2007). Afterwards, the proventriculus alone regulates the rate at which food enters the midgut by peristaltic contractions (Woodring and Lorenz, 2007). The midgut of *G. bimaculatus* contains two large highly folded caeca, which are the main sites of digestive enzyme secretion (Woodring and Lorenz, 2007). The caecal epithelium secrets a mucous fluid, the peritrophic gel (PG), whereas the posterior part of the midgut and the ventriculus produce a type I peritrophic membrane

(PM) (Terra, 2001). The PM is formed by a large number of cells and is associated with the physical distension of the gut by food ingestion (Richards and Richards, 1977; Terra, 2001). The PG differs from PM in its permeability properties and the lack of mechanical resistance, and it also regulates the compartmentalization of digestion, so that the midgut cells are separated from the food when a forward flow of digestive enzymes or caecal fluid into the crop is necessary (Terra, 2001).

The hindgut of *G. bimaculatus* consists of pylorus, ileum, colon and rectum. Typical for most insects the Malpighian tubules enter the gut at the region of the pylorus, which is characteristically located between the midgut and the hindgut. In *G. bimaculatus* the pylorus, and therefore the Malpighian tubules, occur at the posterior end of the ileum. In some insect species (termites, beetles) the ileum forms a fermentation pouch housing bacteria or protozoa to digest otherwise indigestible food particles (e.g. cellulose, lignin) (Gillot, 2005). In *G. bimaculatus* the wall of the ileum also forms a large exoperitrophic pouch with finger-like invaginations filled with bacterial soup, which is held in place by ileal bristles (Woodring and Lorenz, 2007). The main function of the hindgut is the absorption of ions, water and small organic molecules in combination with osmoregulation (Gillot, 2005), but there is also digestion and absorption of some nutrients (Thomas and Nation, 1984; Noble-Nesbitt, 1998).

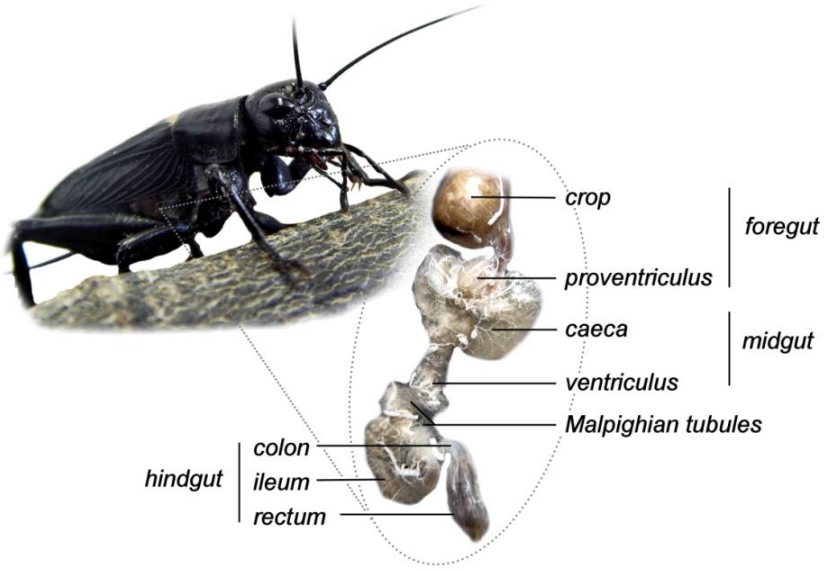

Figure 1: The digestive tract of *Gryllus bimaculatus*.

1.2 Digestive enzymes

Digestive enzymes are hydrolases that split biopolymers by the insertion of water molecules. They mainly act in the midgut, where different types of digestive enzymes (e.g. proteases, carbohydrases, lipases) are secreted by the midgut epithelium. In general the degradation process of biopolymers in the insect midgut is organized in three phases: the hydrolysis of (A)

polymers to oligomers, (B) oligomers to di- or monomers and (C) dimers to monomers that can be absorbed (Terra and Ferreira, 1994).

1.2.1 Proteases

Proteases are enzymes with a wide range of physiological roles, which are required in different vital processes such as digestion, growth, fertilization, immunological reactions, wound healing and cell death (Lazure, 2002; Kanost and Clem, 2012). Proteases are classified in two groups: endopeptidases and exopeptidases. Endopeptidases are able to split large proteins at internal peptide bonds adjacent to the positively charged or nonpolar aliphatic and aromatic groups. These smaller peptides in turn are hydrolysed at the terminal ends to amino acids by exopeptidases (Figure 2). Endopeptidases are very small (~25-30 kDa) and able to pass through the pores of the peritrophic membrane (or gel) in contrast to exopeptidases, which are much larger (>100 kDa) and are bound to the epithelial plasma membrane. There are three subclasses of endopeptidases, which are classified according to their catalytic mechanism and active site: serine proteases, cysteine proteases, and aspartic proteases. Serine proteases for example have a serine and a histidine in their active site. Within the current dissertation the proteolytic activity in the midgut of *G. bimaculatus* was characterized focusing on the serine protease trypsin, which is by far the most important endopeptidase in insects except in some hemipteran and coleopteran species (Wolfson and Murdock, 1990, Terra et al., 1996), and on an aminopeptidase.

Trypsin and its precursor trypsinogen were early targets of protein sequencing studies in vertebrates, and trypsin precursors have also been described in *Aedes aegypti* (Barillas-Mury, et al., 1991; Graf et al., 1991), *Drosophila melanogaster* (Davis et al., 1985), *Simulium vittatum* (Ramos et al., 1993) and *Stomoxys calcitrans* (Moffatt and Lehane, 1990).

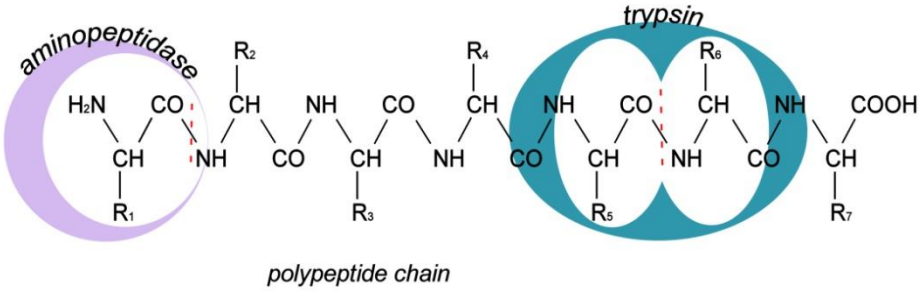

Figure 2: Enzymatic cleavage of a polypeptide chain by aminopeptidase (EC 3.4.11) and trypsin (3.4.21.4). In the case of trypsin R_5 represents the aminoacids lysin or arginine. R_x = aminoacids, ····· cutting site.

1.2.2 Carbohydrases

Carbohydrases catalyse the hydrolysis of polysaccharides into simple sugars, which requires two types of enzymes, endosaccharidases and exosaccharidases. Endosaccharidases cleave the internal bonds of carbohydrates to produce oligosaccharides and disaccharides, which are hydrolysed later on by exosaccharidases to monosaccharides that can be absorbed (Terra et al., 1996).

1.2.2.1 Amylases

α-Amylase (EC 3.2.1.1) represents the most ubiquitous polysaccharidase, that catalyses the initial hydrolysis of starch, glycogen and other polysaccharides to maltodextrin or maltotriose through the cleavage of α-1→4 glucan linkages (Figure 3). Thereafter, those oligosaccharides are digested via β-amylase (EC 3.2.1.2) to maltose followed by hydrolysis via α-glucosidases (EC 3.2.1.20) to glucose suitable for absorption. Although there is a considerable literature on insect amylase activity (Terra and Ferreira, 1994; Nagaraju and Abraham, 1995; Markwick et al., 1996; Terra et al., 1996; Alfonso et al., 1997; Franco et al., 2000, 2002; Titarenko and Chrispeels, 2000; Vinokurov et al., 2007; Valencia-Jiménez et al., 2008; Zibaee et al., 2008; De Sales et al., 2008; Bandani et al., 2009), and also on amylase activity in the lumen of the digestive tract of crickets and related Orthopteroids (Thomas and Nation, 1984; Teo and Woodring, 1985; Colepicolo-Neto et al., 1986; Marana et al., 1997; Woodring et al., 2007, 2009), the enzymatic mechanisms have not been completely elucidated. Different α-amylases seem to be similar in their mechanisms of action, and they contain conserved catalytic residues (Svensson, 1994; MacGregor et al., 2001). α-Amylases are calcium-dependent enzymes, which means that they are stabilized and protected by calcium ions against proteolytic activity and inactivation (Terra and Ferreira, 1994).

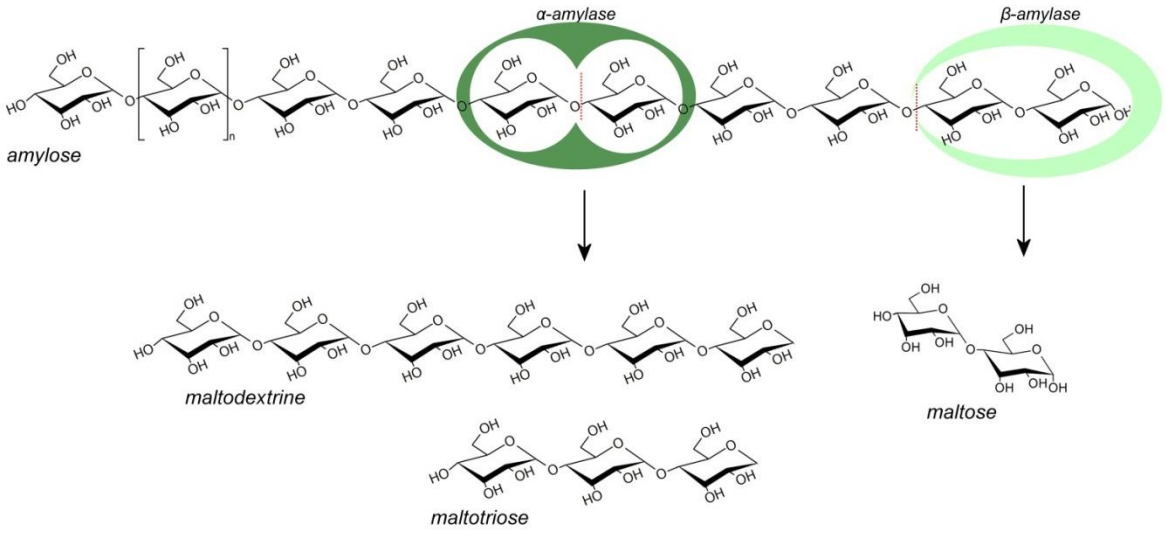

Figure 3: Enzymatic hydrolysis of amylose $(C_6H_{12}O_6)_n$ by amylases.····· cutting site.

1.2.2.2 Cellulases

Cellulose is the most abundant natural organic polymer and the major carbohydrate of plant cell walls, and therefore widely utilized in insect nutrition. Cellulose $(C_6H_{10}O_5)_n$ is a linear polysaccharide of β-1→4 linked glucose residues, and often associated with lignin, pectin or hemicellulose (grasses, wood). Some insect species are well adapted or even specialized (e.g. some beetles, termites) to a high cellulose concentration in their diet. The biodegradation of cellulose in insects was formerly claimed to be a result of symbiontic microorganisms (Martin et al., 1991), but there is increasing evidence for endogenous cellulases (Yokoe and Yasumasu, 1964; Morgan, 1976; Ferreira et al., 1992; Slaytor, 1992; Treves and Martin, 1994; Watanabe and Tokuda, 2001; Lo et al., 2003; Watanabe and Tokuda, 2010, Weidlich et al., 2013).

The digestion of cellulose is accomplished by a system of three cellulolytic enzymes: endo-β-1,4-glucanase (EC 3.2.1.4), exo-β-1,4-cellobiohydrolase (EC 3.2.1.74 and 3.2.1.91), and β-glucosidase (EC 3.2.1.21), which act synergistically to hydrolyse the β-1→4 bonds to glucose monomers (Figure 4). Endo-β-1,4-glucanases hydrolyse cellulose to cello-oligomers by cleaving internal bonds at random. Furthermore, cellulose and cello-oligomers are hydrolysed by exo-β-1,4-cellobiohydrolases from the reducing and the non-reducing end to cellubiose or further cello-oligomers. Finally, β-glucosidases split cellubiose, or also cello-oligomers, to glucose monomers from the non-reducing ends.

Cellulolytic systems have been reported for more than 70 higher animals (including vertebrates), and often the endogenous cellulase activity was distributed according to phylogenetic relationship and not to food consumed (Yokoe and Yasumasu, 1964; Watanabe and Tokuda, 2001). To date insect cellulolytic activity in digestive fluids, dependent and independent of symbiotic microorganisms, has been shown several times (> 60 species from 7 orders) (Wharton and Wharton, 1965; Ishaaya and Plaut, 1974; Martin et al., 1991; Tokuda et al., 1997; Pitman et al., 2003; Oppert et al., 2010), including identification and cloning of insect cellulases and cellulase genes (Watanabe et al., 1997, 1998; Girard and Jouanin, 1999a; Lee et al., 2004, 2005; Wei et al., 2006; Kim et al., 2008; Watanabe and Tokuda, 2010). Since the first discovery of cellulase genes in the termite *Reticulitermes speratus* (Watanabe et al., 1998), cellulase encoding genes from the glycosyl hydrolase family 9 (GHF 9) were identified in further insect orders (Blattaria, Coleoptera, Orthopteroidea, Hemiptera, Phthiraptera, Hymenoptera), which indicates a possible common ancestor of insects with a GHF 9 cellulase gene (Watanabe and Tokuda, 2010).

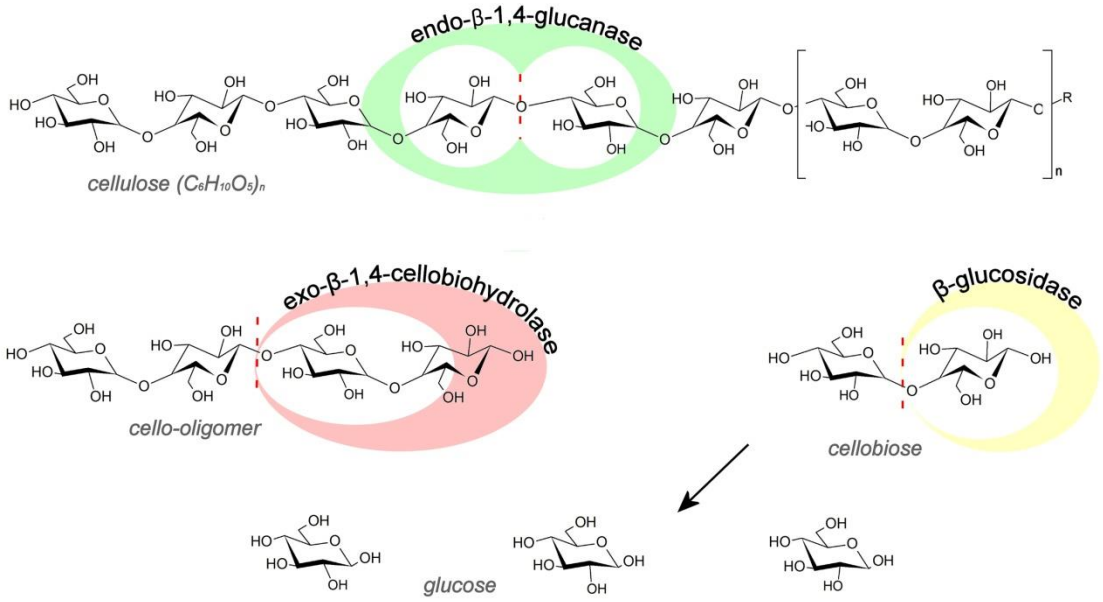

Figure 4: **Schematic view of the enzymatic hydrolysis of cellulose. Biodegradation of cellulose is accomplished by endo-β-1,4-glucanase (EC 3.2.1.4), exo-β-1,4-cellobiohydrolase (EC 3.2.1.74 and 3.2.1.91) and β-glucosidase (EC 3.2.1.21).** ····· cutting site.

1.2.2.3 Chitinases

Chitin is a β-1→4 linked linear polymer of N-acetyl-D-glucosamine (GlcNAc) and a major component of both arthropod exoskeleton and fungi cell walls. In insects chitin is present in the lining of the fore- and hindgut as well as an integral part of insect peritrophic matrix (Terra, 2001; Merzendorfer and Zimoch, 2003).

Insect chitinases are mainly involved in the moulting process. Moulting fluids and venom glands have been well characterized (Kramer and Koga, 1986; Fukamizo and Kramer, 1987; Samuels and Reynolds, 1993; Krishnan et al., 1994; Terra and Ferreira, 1994; Reynolds and Samuels, 1996; Terra et al., 1996.), but many insects also show high expression of chitinase genes and chinolytic activity in the midgut (Shen and Jacobs-Lorena, 1997; Girard and Jouanin, 1999b; Ramalho-Ortigão and Traub-Csekö, 2003; Souza-Neto et al., 2003; Fitches et al., 2004; Bolognesi et al., 2005; Genta et al., 2006). Insect chitinases belong to the glycosyl hydrolase family 18 with a characteristic multi-domain structure. Their molecular mass ranges from 40 to 85 kDa and they vary in their pH optima (pH 4-8) and isoelectric point (pH 5-7) (Arakane and Muthukrishnan, 2010).

Midgut chitinases are involved in dietary digestion, but also in the formation and digestion of the peritrophic membrane and its regulation of thickness and permeability (Shen and Jacobs-Lorena, 1997; Filho et al., 2002; Villalon et al., 2003; Bolognesi et al., 2005). The degradation of chitin requires the action of more than one enzyme type (Figure 5). Chitinases (EC 3.2.1.14) hydrolyse internal bonds of chitin polymers to chitooligomers (chitotetraose, chitotriose,

chitobiose), which are subsequently digested by exo-splitting β-N-acetylglucosaminidases (EC 3.2.1.52) to the monomer GlcNAc (Kramer and Koga, 1986; Reynolds and Samuels, 1996).

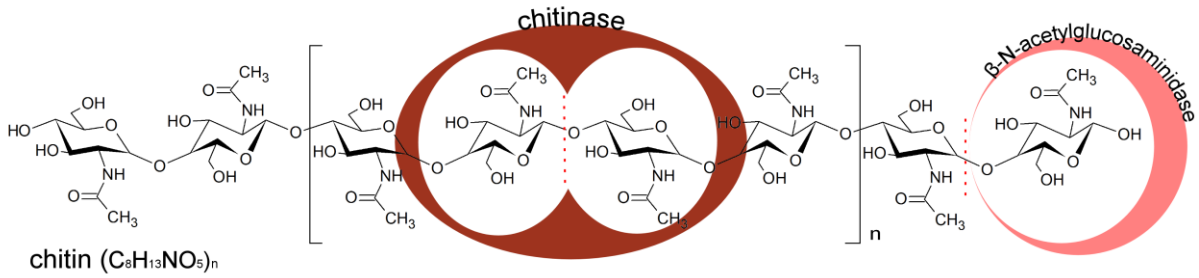

Figure 5: Enzymatic degradation of chitin $(C_8H_{13}NO_5)_n$ by chitinase (EC 3.2.1.14) and β-N-acetylglucosaminidase (EC 3.2.1.52).····· cutting site.

1.2.3 Lipases

Lipids are an important source of energy and essential for insect development, energy storage and oogenesis. Insects have a dietary requirement for polyunsaturated fatty acids during their post-embryonic phases of development, but food requirement differs between species (Dadd, 1983, 1985; Canavoso et al., 2001).

Complete lipid digestion is accomplished by carboxylic ester hydrolases (EC 3.1.1: lipases, esterases, phospholipase A and B) (Figure 6), phosphoric monoester hydrolases (EC 3.1.3: phosphatases) and phosphoric diester hydrolases (EC 3.1.4: phospholipase C and D) (Terra et al., 1996). Thereby, lipases are essential compounds of the fat metabolism and hydrolyse the outer ester links of triacylglycerols from the α-position stepwise to diacylglycerols, monoacylglycerols, glycerol and free fatty acids (Bollade et al., 1970; Hoffman and Downer, 1979; Secundo et al., 2006).

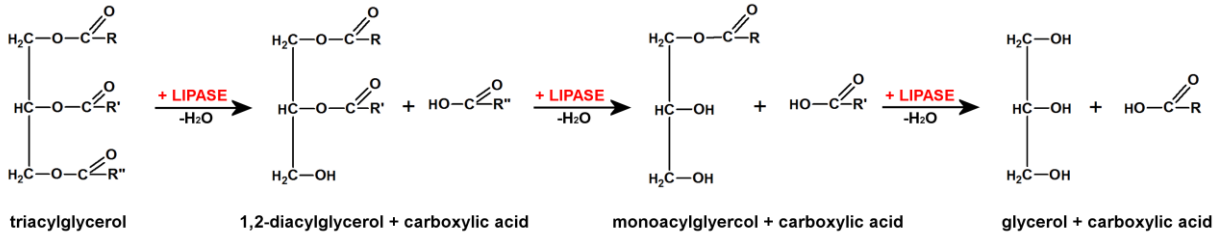

Figure 6: Stepwise hydrolysis of triacylglycerol to glycerol and carboxylic acids.

1.3 Secretory processes for digestive enzymes

Different mechanisms of synthesis, storage and release of digestive enzymes at the cellular level have been described in various insect species (Cristofoletti et al., 2001; Ferreira et al., 2002; Terra et al., 1996; Weidlich et al., 2012). Digestive enzymes are synthesized in the rough endoplasmatic reticulum, processed in the Golgi complex, packed into secretory vesicles and secreted by the gut endothelium via exocytosis, apocrine or microapocrine processes (Terra and Ferreira, 2012) (Figure 7).

In insects, most enzyme release is by exocytosis, and less often by apocrine secretion (Terra and Ferreira, 1994), depending on the midgut region, the enzyme and the species (Graf et al., 1986; Santos et al., 1986; Jordão et al., 1996, 1999; Cristofoletti et al., 2001; Ferreira et al., 2002). During exocytosis enzymes are stored in vesicles, which fuse with the plasma membrane and release their content without any loss of cytoplasm (Figure 7A), whereas apocrine secretion involves a loss of apical cytoplasm following vesicle release, in which the enzymes are stored (Figure 7B). Microapocrine secretion is a common type of apocrine secretion (De Priester, 1971; Heinrich and Zebe, 1973; Nopanitaya and Misch, 1974; Lehane, 1976; Humbert, 1979; Santos et al., 1984; Terra et al., 1988), in which the loss of cytoplasm is minimal and small budding double membrane vesicles (Figure 7C) or pinched-off secretory vesicles are released (Figure 7D). The content of those vesicles is freed by membrane fusion or solubilisation in the midgut lumen (Terra and Ferreira, 2012).

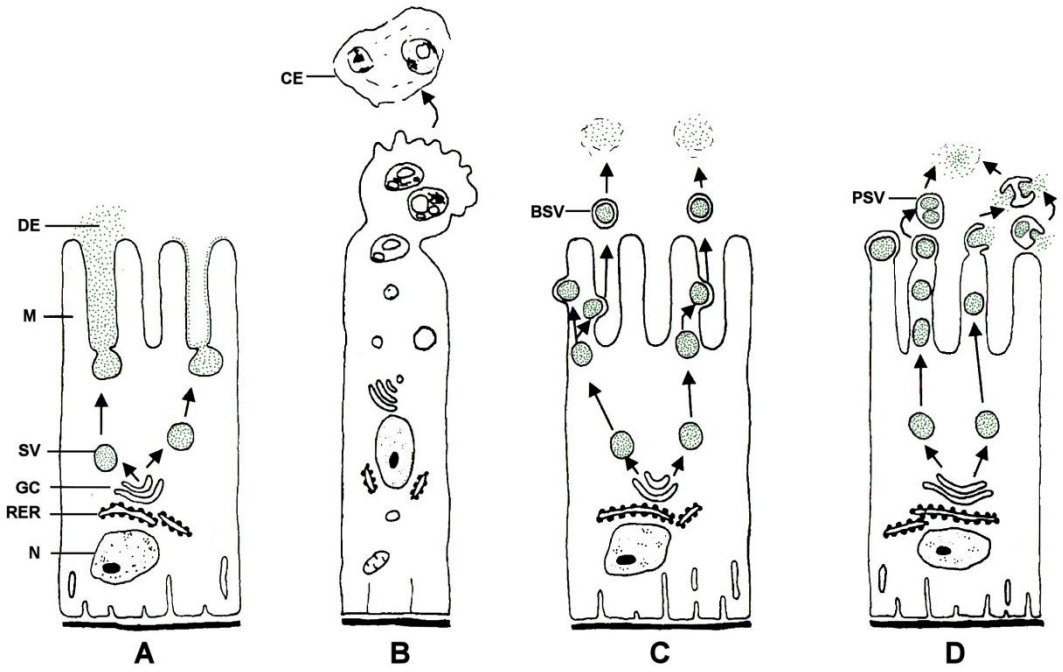

Figure 7: Models for secretory processes of insect digestive enzymes: exocytic secretion (A), apocrine secretion (B), microapocrine secretion with budding vesicles (C) and with pinched-off vesicles (D). BSV: budding secretory vesicle, CE: cellular extrusion, DE: digestive enzymes; GC: Golgi complex, M: microvilli; N: nucleus, PSV: pinched-off secretory vesicles, RER: rough endoplasmatic reticulum, SV: secretory vesicle. (adapted from Terra and Ferreira, 2009)

1.4Regulation mechanisms controlling digestive enzyme secretion in insects

According to their feeding behaviour insects can be classified in two major groups: continuous and discontinuous feeders. Continuous feeders have a continuous stream of food passing through the intestine, while discontinuous feeders (carnivores, haematophagous) have periods where the gut is filled or empty (Lehane et al., 1996). Therefore, discontinuous feeders need a regulation of enzyme secretion. The midgut is the main site of digestive enzyme release and metabolite absorption in insects (Dow, 1992; Chapman, 1998), whereby the control of digestive enzyme secretion depends on various mechanisms (e.g. hormonal, paracrine, prandial) (Lehane et al., 1995) and requires separate regulation of enzyme synthesis and enzyme secretion (Blakemore et al., 1995).

Food consumption plays a fundamental role in the secretion of digestive enzymes, in that not only food intake (Engelmann, 1969; Dadd, 1970; Chapman, 1998) but also the composite of the nutrition regulates secretion mechanism (prandial release mechanism) (Chapman, 1985; Terra, 1990; Lehane et al., 1996; Terra et al., 1996). Thereby, small components of the diet (different nutrients) interact directly with the secretory cells of the midgut and stimulate the secretion of specific digestive enzymes (Lehane et al., 1995).

Although some studies already reported the influence of hormones on enzyme secretion in the midgut of insects (Applebaum, 1985; Chapman, 1985), it is still controversial whether the hormone system has a direct influence on digestive enzyme release or rather changes in hormone systems are subsequent due to the fact of treatment (Lehane et al., 1996).

The insect midgut epithelium contains large numbers of endocrine cells (Montuenge et al., 1989; Endo et al., 1990; Sehnal and Žitňan, 1996), which likely play a role in intestinal activities (Lehane et al., 1996). These cells have a hemolymph side and a gut lumen side. In this model, nutrient receptors on the lumen side can stimulate the release of paraneurohormones into the hemolymph, which bind to receptors and induce the release of digestive enzymes into the gut lumen (Figure 8).

Several neuropeptides including FMRFamide-related peptides, proctolin, insect kinins and allatoregulatory peptides have already been identified in the enteric nervous system and in the endocrine cells of the gut (Reichwald et al., 1994; Yu et al., 1995; Sehnal and Žitňan, 1996), and were shown to affect food uptake, gut motility (Wei et al., 2000; Predel et al., 2001; Aguilar et al., 2004; Meyering-Vos and Müller, 2007a; Meyering-Vos and Woodring, 2008; Audsley and Weaver, 2009) and the release of digestive enzymes in particular (Fusé et al., 1999; Harshini et al., 2002a,b; Aguilar et al., 2003; Hill and Orchard, 2005; Sakai et al., 2006; Audsley and Weaver, 2009; Woodring et al., 2009; Lwalaba et al., 2010a).

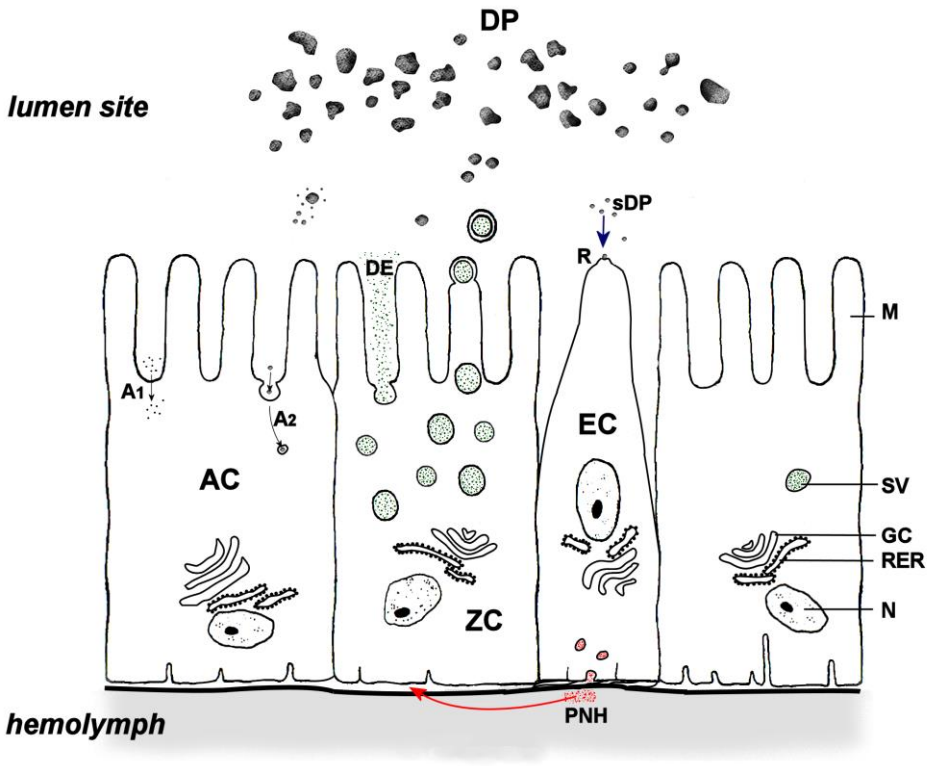

Figure 8: Regulation mechanism for digestive enzyme release: small digestive products bind to receptors of endocrine cells on the midgut side to free paraneurohormones, which stimulate the enzyme release of zymogene cells (paracrine mechanism). A1: absorption of soluble compounds, A2: absorption of small digestive products via endocytosis, AC: absorptive cell, DE: digestive enzymes (green), DP: digestion products, EC: endocrine cell, GC: Golgi complex, M: microvilli, N: nucleus, PNH: paraneurohormones, R: receptor, RER: rough endoplasmatic reticulum, sDP: small digestive products, SV: secretory vesicles, ZC: zymogen cell. (modified from Terra and Ferreira, 2009)

1.4.1 Allatostatins

Allatoregulating peptides are divided in two groups based on their stimulatory (allatotropins) or inhibitory (allatostatins) effect on juvenile hormone biosynthesis in the corpora allata (CA) (Hoffmann et al., 1999; Gäde, 2002). Allatostatins belong to a well-documented group of neurohormones, which has been identified in a large number of insect species (Stay, 2000), and can be classified in three subgroups according to their sequence homology: allatostatin A (AST-A, FGLamides), allatostatin B (AST-B, W(X_6)Wamides) and allatostatin C (AST-C, PISCF-OH) (Stay, 2000; Meyering-Vos et al., 2001; Hoffmann, 2003).

The Allatostatin type A peptide is characterized by a common C-terminus sequence Tyr/Phe-Xaa-Phe-Gly-Leu-Ile/Val-amid (Stay et al., 1991) and was first identified from *Diploptera punctata* (Woodhead et al., 1989; Donly et al., 1993) and other cockroach species (Ding et al., 1995; Bellés et al., 1999, Bendena et al., 1999). Due to their pleiotropic function, AST-A peptides are expressed in different tissues (Stay, 2000). In *G. bimaculatus* the AST-A gene is

strongly expressed in the brain, the suboesophageal ganglion and the caeca of the digestive tract (Meyering-Vos and Hoffmann, 2003). The prohormone precursor encodes for 14 putative *Gryllus*-AST-A peptides which are interspaced by acidic spacers (Meyering-Vos et al., 2001) (Figure 9A).

An inhibitory effect of AST-A peptides on JH biosynthesis was demonstrated for cockroaches, termites, crickets and some beetles (Stay and Tobe, 2007; Abdel-latief and Hoffmann, 2010). But AST-A peptides have also myoinhibiting effects on different parts of the insect gut or on the oviduct (Gäde and Hoffmann, 2005). Moreover, they inhibit the production and release of vitellogenin from the fat body of cockroaches (Martín et al., 1996, 1998) and affect the secretion of digestive enzymes (Fusé et al, 1999; Aguilar et al., 2003; Sakai et al., 2006; Digali et al., 2010).

1.4.2 Sulfakinins

Sulfakinins (SK) are another family of neuropeptides with myotropic function, which were primarily isolated from the cockroach *Leucophaea maderae* (Nachman et al., 1986a). Insect SKs show structural homology to the peptides gastrin and cholecystokinin, which are involved in the regulation of food uptake in vertebrates (Nachman et al., 1986b). SKs are characterized by a highly conserved C-terminal hexapeptide sequence DY(SO_3H)GHMRF-$NH_2$and a sulphated tyrosine residue (Audsley and Weaver, 2009).SKs were isolated from several different insect species (Veenstra, 1989; Schoofs et al., 1990; Nichols et al., 1988, 1992; Fonagy et al., 1992; Duve et al., 1995; East et al., 1997; Maestro et al., 2001; Meyering-Vos and Müller, 2007b) and were shown to affect food uptake and enzyme release into the digestive tract (Nachman et al., 1997; Wei et al., 2000; Maestro et al., 2001; Harshini et al., 2002b; Schoofs and Nachman, 2006; Downer et al.,2007; Meyering-Vos and Müller, 2007a; Meyering-Vos and Woodring, 2008).

In *G. bimaculatus* the SK prohormone precursor encodes two SK peptides (Figure 9B), which both show a strong expression only in the brain (Meyering-Vos and Müller, 2007b).

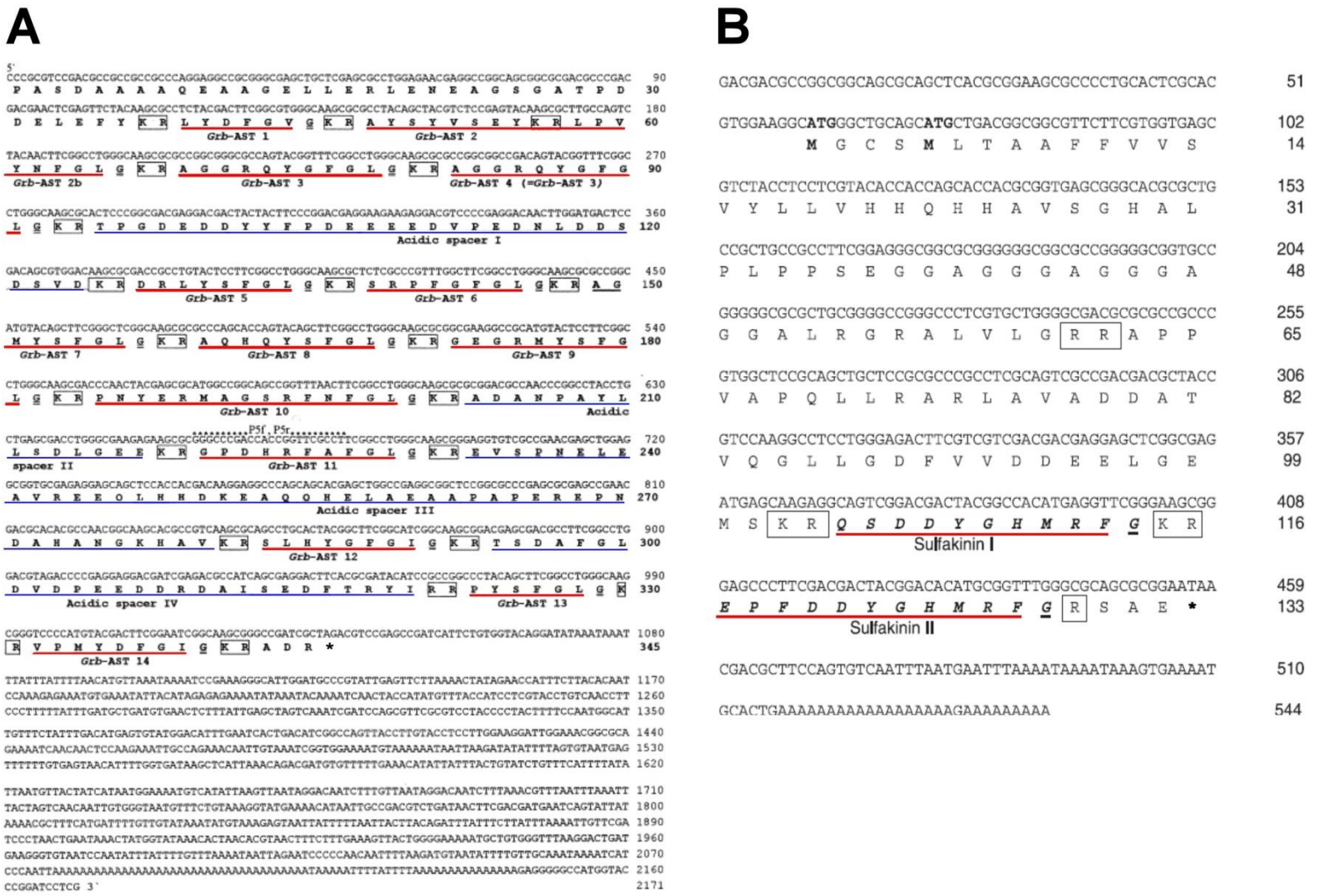

Figure 9: **Nucleotide sequence of the allatostatin (A) and sulfakinin (B) precursor cDNA of *G. bimaculatus* and the deduced amino acid sequences of the preprohormone polypeptides**. Adapted from (A) Meyering-Vos et al. (2001), (B) Meyering-Vos and Müller (2007b). The cDNA sequence is numbered from the most distal nucleotide identified on the 5' end. The deduced protein sequence is in boldface. Potential cleavage sites are boxed. Neuropeptide sequences are underlined red, those of acidic spacers blue, preceding glycine residues (required for α-amidation) black. * represents the stop codon.

1.5 Enzyme inhibitors

1.5.1 Plant protease inhibitors

Over recent years the adaptation mechanisms of insects to their nutrition and abiotic environment have become more important with view of the increasing number of pest insects and their control. Therefore, a lot of studies focused on the investigation of insect digestion and a probable application of natural enzyme inhibitors for pest control.

Many plants produce protease inhibitors (PI) as a defence mechanism against feeding damage (Fan and Wu, 2005). PIs inhibit the proteases present in the midgut lumen and crop of insects (Johnston et al., 1993; Telang et al., 2005; Duncan et al., 2006; Brioschi et al., 2007), but also affect the secretion of proteases by the epithelium (Lwalaba et al., 2010b; Weidlich et al., 2012). While PIs were thought to have the potential to protect plants against herbivorous insects (Broadway and Duffey, 1986; Broadway et al., 1986; Hilder et al., 1987; Johnson et al., 1989; Oppert et al., 1993; Orozco-Cardenas et al., 1993; McManus et al., 1994), it was soon apparent that insects have evolved different strategies to deal with PIs in the diet: (a) enzyme hyperproduction (Broadway and Duffey, 1986; Johnston et al., 1993; Broadway, 1995, Hivrale et al., 2011), (b) up- and down-regulation of proteases (Jongsma et al., 1995; Cloutier et al., 2000; Zhu-Salzman et al., 2003; Brioschi et al., 2007; Dunse et al., 2010a, b), (c) increasing release of inhibitor-insensitive enzyme isoforms (Jongsma et al., 1995, 1996; Paulillo et al., 2000; Brito et al., 2001; Volpicella et al., 2003; Brioschi et al., 2007; George et al., 2008; Hivrale et al., 2011; de Oliveira et al., 2013) or (d) secretion of PI-degrading proteases (Jongsma et al., 1996; Michaud, 1997; Girard et al., 1998).

The Kunitz type trypsin inhibitor from soybean (SBTI) is a small protein (~25 kDa) which interacts with trypsin-like proteases by forming an irreversible complex with a very low dissociation constant and, therefore, blocking the active site of the enzyme (Kunitz, 1948). Furthermore, SBTI is quite resistant to higher temperatures by changing conformation (Kunitz, 1948). The inhibitory effect of SBTI against midgut proteases was demonstrated for different insects *in vitro* and *in vivo* (Applebaum et al., 1963; Miller et al., 1974; Christeller et al., 1990; Johnston et al., 1993; Oppert et al., 2005; Lwalaba et al., 2010b; Weidlich et al., 2012).

1.5.2 Endogenous protease inhibitors

Endogenous serine protease inhibitors (EPI) are widely found in all tissues of all animals. The serpins, a very large class of proteases, are mostly intracellular, and undergo a unique change in shape when they inhibit target proteases (Huntington et al., 2000). They regulate such processes as coagulation, inflammation, and immunity. However, some insect inhibitors involved with immune responses (coagulation, activation of phenoloxidases) belong to the classical Kunitz and Kazal type inhibitors (Kanost, 1999). They bind to the active site of the

proteases and block access (Kunitz, 1948). Protease inhibitors of the Kazal type are found in the salivary gland and saliva of *Nauphoeta cinerea*, which are particularly effective against the bacterial protease subtilisin. This suggests a defensive mechanism against the masses of bacteria typically found in the cockroach crop, some of which are probably pathogenic (Taranushenko et al., 2009). Inhibitory peptides in the midgut lumen are less well studied, though trypsin and subtilisin inhibitors are described from whole midgut preparations of the cockroach *N. cinerea* (Elpidina et al., 2001) and in five additional cockroach species (Vinokurov et al., 2007). Inhibitors from the cockroach gut act on endogenous digestive proteinases and may represent a new mechanism of digestion regulation.

1.5 Research gaps

Although, a lot of studies focused on the digestion in insects, there is little knowledge on the factors controlling enzyme secretion (Lehane et al., 1996; Blakemore et al., 1995; Woodring et al., 2009; Lwalaba et al., 2010a). Therefore, the present dissertation focuses on the determination of the effect of different endogenous (organismic) and exogenous (environmental) factors on the regulation of digestive enzyme secretion into the midgut of the two-spotted field cricket *G. bimaculatus* (Figure 10). In particular, the following experiments were carried out:

- ***Sample type***: Enzyme activity in different sample types (luminal content, tissue incubation medium, tissue homogenate)
- ***Age-dependent enzyme release***: Changes in enzyme release of female and male *G. bimaculatus* from last larval instar to adult stadium
- ***Neuropeptides***: The effect of cricket allatostatin type A and sulfakinin on the release of digestive enzymes using *in vitro* incubation and RNA interference
- ***Calcium ions***: Dependency of enzyme release on the presence or absence of calcium ions in tissue incubation medium
- ***Trypsin zymogen***: The presence and activation of a putative trypsin precursor, and the autolysis of *Gryllus*-trypsin
- ***Endogenous protease inhibitors***: The presence of endogenous protease inhibitors in the midgut of adult crickets
- ***Temperature***: The effect of incubation and rearing temperature on enzyme release with respect to a putative temperature acclimation
- ***Light-dark cycle***: Food uptake and enzyme release during photo- and scotophase within 24 h in penultimate instar, last larval instar and adult stadium
- ***Food***: The effect of feeding and starvation, and the influence of various nutrients (*in vitro* and *in vivo* studies) on enzyme secretion
- ***Plant protease inhibitors***: Dose-dependent inhibition of trypsin secretion and activity caused by feeding protease inhibitor (SBTI) enriched diets in adult *G. bimaculatus*

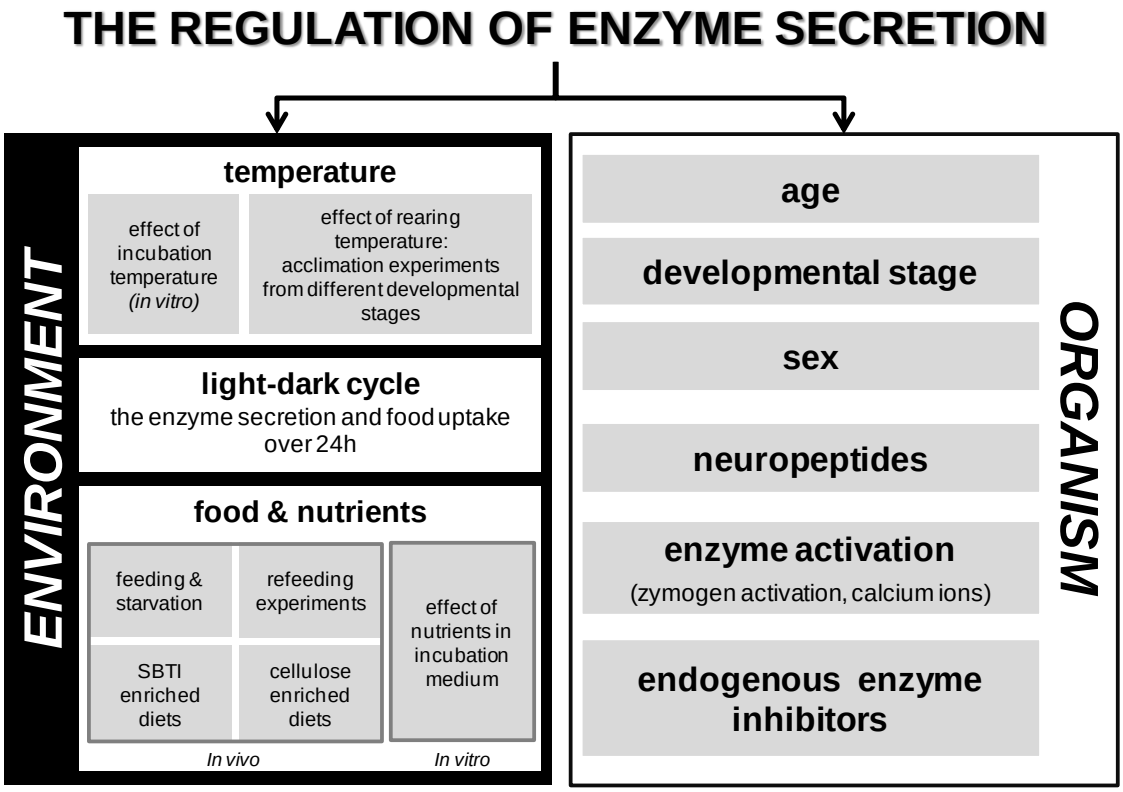

Figure 10: Experimental overview: determination of the effects of endogenous (organism) and exogenous (environment) factors controlling the regulation of enzyme secretion in *G. bimaculatus.* *Note: not all experiments were carried out for all enzymes. For details, please check the corresponding articles.*

2 Synopsis

The focus of this dissertation was on the determination of different factors regulating the secretion and activity of digestive enzymes in *G. bimaculatus.* The factors that regulate the enzyme release are essential for the adaptation of an insect to its environment. Therefore, different endogenous and environmental factors have been analysed on their effects on representatives of the three enzyme groups (1) proteases (publication 1 & 4), (2) polysaccharidases (publication 2) and (3) lipases (publication3).
For a clearer overview, the following text is organized according to the studied factors, and not to the type of enzymes, as in the publications.

2.1 Enzyme assays, kinetic parameters and sample preparation

2.1.1 Enzyme activity assays

The activity of trypsin and aminopeptidase were determined by the amount of *p*-nitroaniline (*p*NA) released in one minute from the synthetic substrates N_α-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (Ba*p*Na) (Sigma) and L-leucine *p*-nitroanilide hydrochloride (L*p*Na) (Sigma, Germany), respectively, measured at 405 nm. Lipase activity was determined by the amount of *p*-nitrophenol (*p*NP) released from *p*-nitrophenyl palmitate (*p*NPP) (Sigma, Germany) within 30 min, measured at 410 nm. Amylase activity was determined by the amount of maltose split from starch during 30 min incubation at 37°C using dinitrosalicylic acid reagent (DNS) to detect the reducing sugars at 530 nm (Bernfeld, 1955). Cellulase activity was measured using two different methods. Method 1 used DNS to detect the amount of cellubiose split from carboxymethyl cellulose (CMC). Method 2 measured cellulase activity using EnzChek® cellulase fluorescent substrate (Life Technologies) at 360 nm excitation and 460 nm emission and was described as relative fluorescent units [RFU] after 30 min incubation at 25°C. Chitinase activity was determined as the amount of remazol brilliant violet (RBV) released from the substrate carboxymethyl-chitin-remazol brilliant violet (CMCh) (Hornik, Germany) within one hour, measured at 550 nm. The concentration of soluble protein in the samples was measured at 595 nm with the Bradford protein assay using Roti-Quant® (Roth, Germany).

2.1.2 Kinetic parameters

The kinetic parameters (temperature and pH optima, K_m and V_{max} values) of trypsin, cellulase, chitinase and lipase (and the corresponding substrates) were determined and the data for aminopeptidase and amylase were taken from Woodring et al. (2009) (Table 1). The secretion from caecal tissue was almost linear for all tested enzymes within the first 30 min incubation at 37°C in low glucose Ringer (LGR) (10 mg glucose/100 ml *Gryllus* Ringer).

Table 1: Temperature and pH optima, K_m and V_{max} values of digestive enzymes from the midgut of *G. bimaculatus*.

	Temperature	pH	K_m	V_{max}
Aminopeptidase[1,*]	> 35°C	> 8.1	1.1 mM L*p*Na	0.25 mmol·min^{-1}
Trypsin [2]	40°C	8.1	0.4 mM BA*p*NA	78.12 nmol *p*NA·min^{-1}·caeca^{-1}
Amylase[3,*]	>35°C	6.2	21 mg starch·mL^{-1}	2.8 µg maltose·min^{-1}
Cellulase [3]	40°C	5.0	0.357 % CMC	9 µg cellubiose·min^{-1}
Chitinase [4]	40°C	9.0	0.62 µg·µL^{-1} CMCh	1.66 µg RBV·µL^{-1}
Lipase [5]	37°C	8.5	0.4 mM *p*NPP	29.26 nmol *p*NP·min^{-1}

[1] L*p*Na = L-leucine *p*-nitroanilide hydrochloride, [2] Ba*p*Na= N_α-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride, [3] DNS = dinitrosalicylic acid reagent, [4] CMCh = carboxymethyl-chitin-RBV 5R, [5] *p*NPP = *p*-nitrophenyl-palmitate. CMC = carboxymethyl cellulose, *p*NA = *p*-nitroaniline, *p*NP = *p*-nitrophenol, RBV = remazol brilliant violet.
[*] adapted from Woodring et al. (2009)

2.1.3 Enzyme activity in different sample types

In order to test the influence of different factors on enzyme activity and secretion, three sample types were analysed: (1) lumen content, (2) tissue homogenate and (3) tissue incubation medium.

(**1**) ***Lumen content***: A defined amount of caecal luminal content was diluted in *Gryllus* Ringer (8 g NaCl, 0.4 g KCl and 0.4 g $CaCl_2$ per litre brought to a pH of 7.2 with 1 g Hepes) with few crystals of N-phenylthiourea (PTH). The sample was mixed and centrifuged at 11,000 g for 2 min at 4°C and the supernatant was used for enzyme assays. The enzyme activity in the caecal luminal content is an estimate of the amount of enzymes that accumulate at a given age and time. The PTH inhibits the enzyme phenoloxidase, which otherwise leads to an increasing absorbance of the sample and, therefore, cannot be analysed by photometric assays or sophisticates the results (e.g. changes in absorbance per minute).

(2) ***Tissue homogenate***: The enzyme activity in tissue homogenate represents the amount of enzymes stored in tissue cells and, therefore, represents an indicator of enzyme synthesis rate. The caeca were removed, cut opened in a flat-sheet gut preparation (Blakemore et al., 1995) and rinsed three times with *Gryllus* Ringer. The rinsed tissue was transferred to fresh *Gryllus* Ringer containing few crystals of PTH and homogenized at the lowest setting for few seconds with ultrasonicator (Branson Sonifier 250). The sample was centrifuged at 11,000 × g for 10 min, the pellet discarded and the supernatant was used for enzyme assays.

(3) ***Tissue incubation medium***: In order to analyse the release of digestive enzymes (or precursors) from the caecal tissue the caeca were removed, cut opened to a flat-sheet preparation (Blakemore et al., 1995) and rinsed 3-times with *Gryllus* Ringer. Afterwards, the caecal tissue was incubated in LGR for 30 min at 37°C without shaking. The air-filled trachea kept the caeca at the surface of the medium. Following incubation, the caecal tissue was discarded and relatively few separated cells were removed by centrifugation at low speed (2,000 × g) for 2 min at 4°C.

The activities of amylase, cellulase, trypsin, aminopeptidase and lipase were analysed in samples of lumen content, tissue homogenate and tissue incubation medium (Figure 11) (publication 1-3).

Amylase activity was higher in the luminal content of both female and male crickets compared to tissue incubation medium, but there was also high activity in tissue homogenate (Figure 11A). In contrast, cellulase showed high activity in tissue incubation medium (Figure 11A). Protease activity was higher in lumen content than in tissue homogenate, but aminopeptidase also showed very high activity in tissue incubation medium (Figure 11B). Lipase activity was always highest in luminal content, but in female crickets there was also increased activity in tissue homogenate (Figure 11C).

In general, the activity of all tested enzymes indicated the same trend in both sexes. High activity in tissue incubation medium illustrated an ample enzyme release from caecal tissue, while increasing activity in tissue homogenate indicated a large store of vesicles containing active enzymes, as measured for amylase (Figure 11A) and lipase in female crickets (Figure 11C).

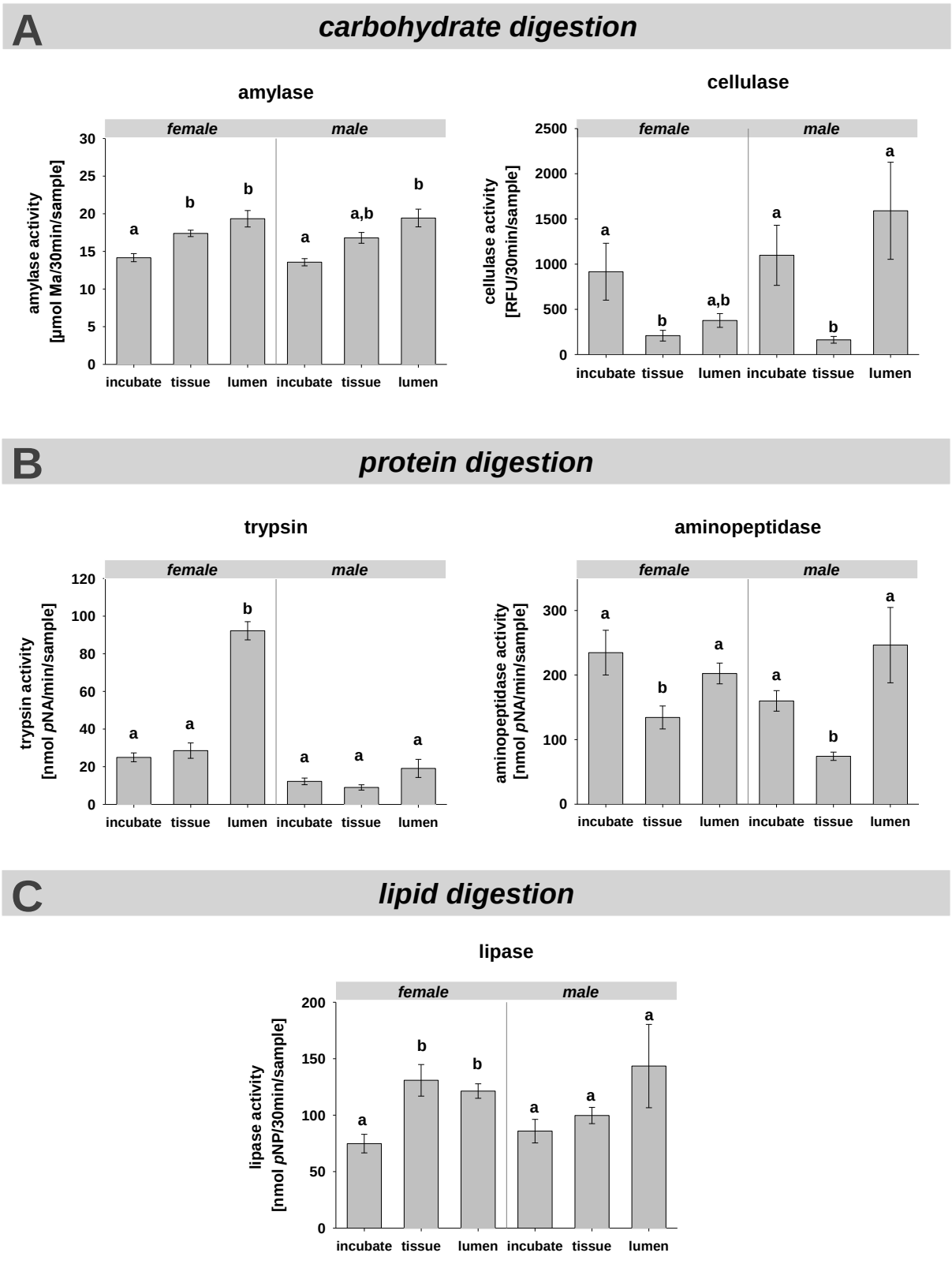

Figure 11: Activity of (A) carbohydrases (amylase and cellulase), (B) proteases (trypsin and aminopeptidase) and (C) lipase in samples of tissue incubation medium (incubate), tissue homogenate (tissue) and luminal content (lumen) of 2-day-old female and male *G. bimaculatus*. Mean ± SEM. n = 10. Statistics: Kruskal-Wallis test and post hoc Dunn's test. Different letters indicate significant differences in enzyme activity between the sample types.

2.2 The effect of endogenous factors

Endogenous factors play an important role in feeding and digestion. Therefore, the age-dependency of enzyme release in *G. bimaculatus* was analysed throughout last larval instar and the first days of adult life for all major digestive enzymes (carbohydrases, proteases, lipase).

The influence of neuropeptides on enzyme secretion was tested by *in vitro* incubation of caecal tissue in LGR containing the peptide AST-5 (allatostatin type A), and *in vivo* effects of allatostatins and sulfakinins were investigated using RNA interference inducing gene suppression.

The secretion mechanism of enzymes was determined by adding calcium ions into the incubation medium. Furthermore, the endoprotease trypsin was studied for autolysis, activation and the presence of a putative precursor.

2.2.1 Age-dependent enzyme release

The secretion of trypsin (publication 1), amylase, cellulase and chitinase (publication 2), lipase (publication 3) and aminopeptidase was followed in male and female crickets from moult to last instar larvae until day ten of the adult stadium (Figure 12).

The secretion of amylase, cellulase, lipase, trypsin, and aminopeptidase was very similar in both sexes. In last instar larvae the release of the enzymes increased until day six followed by a decrease 48-72 h prior to the final moult. After the imaginal moult enzyme release increased, reaching a maximum activity on day 2-4 of the adult stage. The secretion of amylase, cellulase, lipase, trypsin, and aminopeptidase corresponds closely to the daily feeding rate of adult crickets (Woodring and Lorenz, 2007). From these results we conclude that enzyme release through all developmental stages is strongly influenced by food uptake. In general, the secretion of lipase, trypsin, and aminopeptidase is higher in female crickets, while cellulase secretion is higher in males. The release of amylase and chitinase was nearly equal in both sexes.

In contrast to other digestive enzymes chitinase secretion is correlated to the moulting process and always highest at the day of moult. Chitinase in the midgut of *G. bimaculatus* functions primarily in the digestion of the cuticular lining of the foregut and cannot be considered as a major digestive enzyme in food utilization.

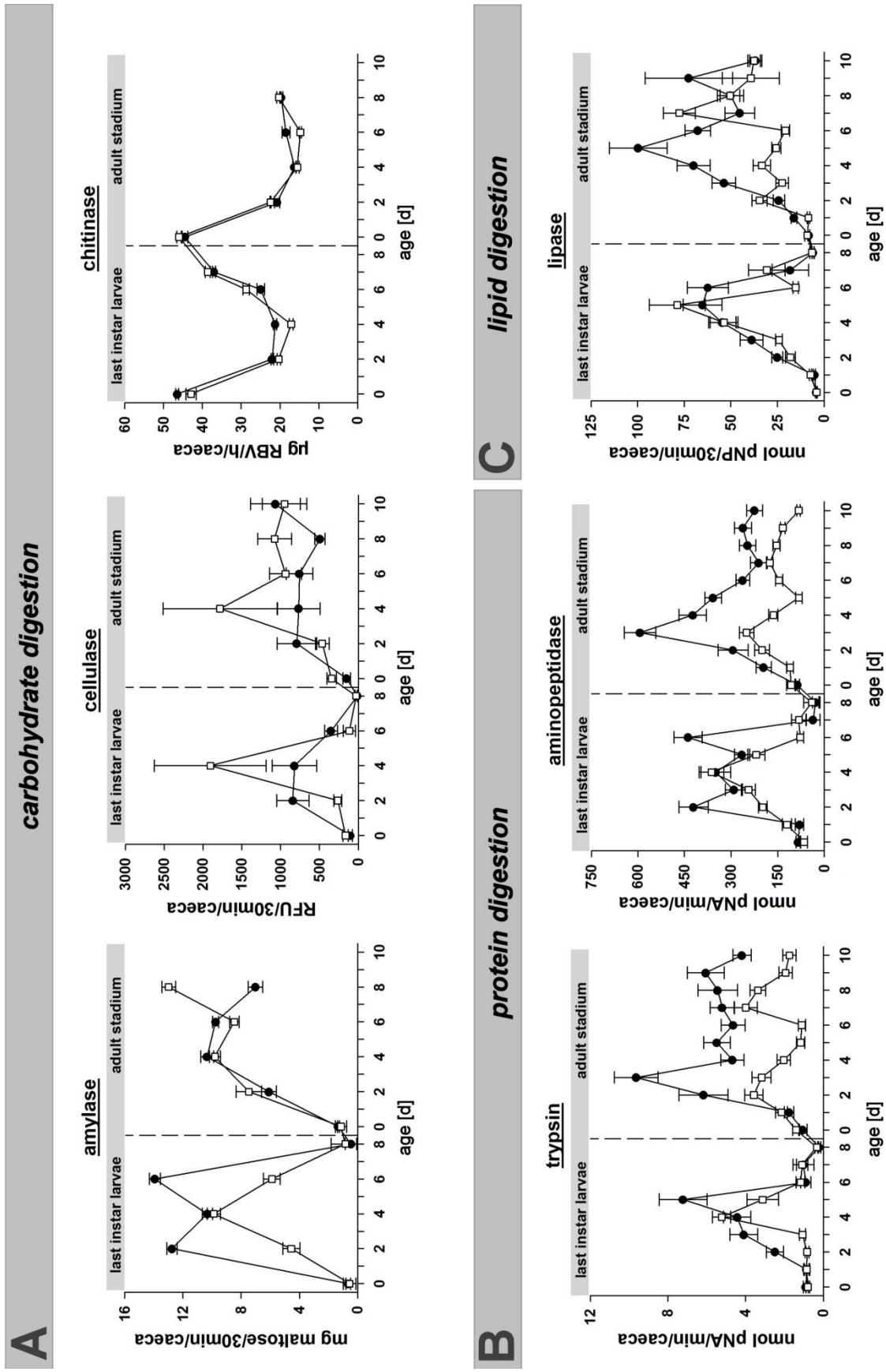

Figure 12: **Age-dependent activity of (A) carbohydrases, (B) proteases and (C) lipase in the caecal secretion medium (30 min incubation at 37 °C) of male (□) and female (●) last instar larvae and adults of *G. bimaculatus*.** Mean ± SEM. n = 10-37.

2.2.2 Neuropeptides

2.2.2.1 Allatostatin type A

The allatostatin type A peptide and its gene, which is strongly expressed in brain, suboesophageal ganglion and caeca of *G. bimaculatus* (Meyering-Vos and Hoffmann, 2003), were analysed with regards to their influence on digestive enzyme release using *in vitro* and *in vivo* studies. AST-5 (DRLYSFGK-NH_2) (Bachem, Germany) was used for *in vitro* incubation of caecal tissue following enzyme activity assays and quantification of soluble proteins. RNA interference was used to analyse the gene function of allatostatin by gene silencing *in vivo,* a method already employed for allatostatin peptides in this species by Meyering-Vos et al. (2006).

In vitro studies

The influence of AST-5 *in vitro* on enzyme release of amylase, trypsin, and aminopeptidase has already been reported by Woodring et al. (2009), where amylase and trypsin release increased during incubation with 10^{-8} to 10^{-5} M AST-5. The data of the current study did not fully confirm these results. In this study, the *in vitro* effect of AST-5 on enzyme release from caeca was tested by addition of appropriate dilution of 10^{-3} M stock solution of AST-5 (in 20 % acetonitrile) in LGR. The incubation medium was tested for enzyme activity and concentration of soluble proteins. There was a trend of decreasing release of soluble proteins in response to higher concentration of AST-5 (Figure 13).

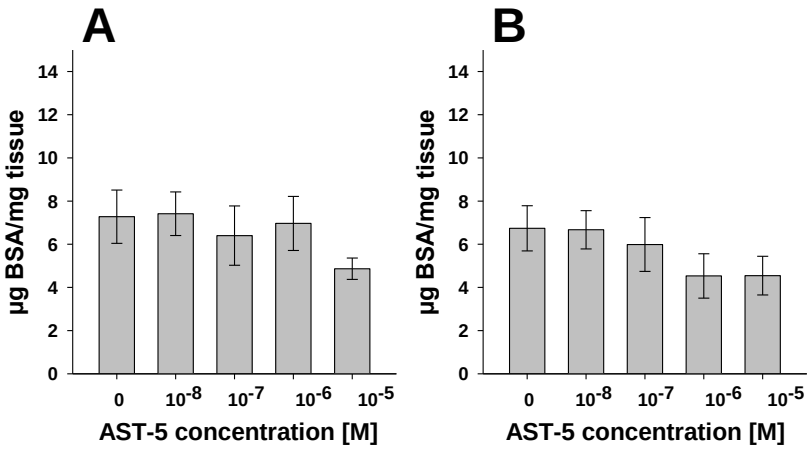

Figure 13: ***In vitro*** **effect of AST-5 on the amount of protein [µg BSA/mg tissue] released from (A) female and (B) male caecal tissue to incubation medium.** Mean + SEM. n = 10. Statistics: Kruskal-Wallis test. P > 0.05.

Although there were no statistically significant differences, the secretion of amylase and cellulase (Figure 14A), aminopeptidase and trypsin (Figure 14B), and lipase (Figure 14C) in both male and female *G. bimaculatus* showed a continues trend of increased enzyme release in response to higher concentrations of AST-5 (10^{-6} to 10^{-5} M) in incubation medium.

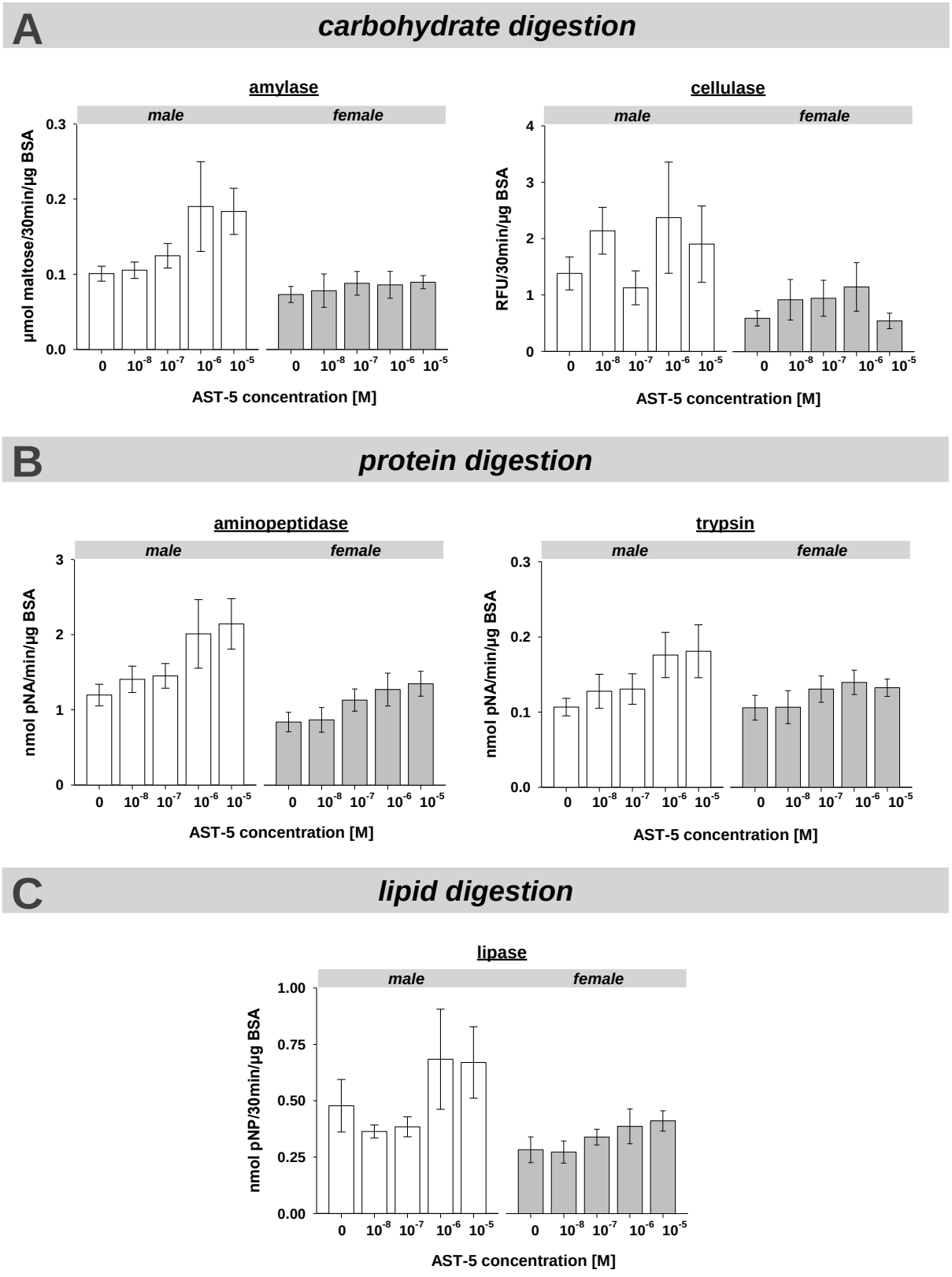

Figure 14: ***In vitro*** **effects of AST-5 on (A) carbohydrases, (B) proteases and (C) lipase secretion from caecal tissue of 2-day-old adult** ***G. bimaculatus*** **males (white) and females (grey).** Mean + SEM. n = 10. Statistics: Kruskal-Wallis test. P > 0.05; no significant differences.

In vivo studies - RNA interference

In vivo effects after injection of dsRNA targeted against AST-A into newly moulted crickets (last instar larvae and adults), following the degradation of the specific mRNAs, were studied by measuring the body weight gain, and the activity of amylase, aminopeptidase, trypsin and lipase in caecal lumen content, tissue homogenate and tissue incubation medium two days after dsRNA injections. The AST-A dsRNA was generated as previously described by Meyering-Vos et al. (2006). 2 to 6 µg AST-A dsRNA in 10 µl *Gryllus* Ringer were injected once with a 100 µl Hamilton syringe between the third and the fourth abdominal segment. Control crickets were injected with *Gryllus* Ringer only.

The body weight gain of crickets injected with AST-A dsRNA did not differ from those injected with Ringer solution (Figure 16 & Figure 15).This corresponds to the results of Meyering-Vos et al. (2006), where differences in body weight gain following AST-A dsRNA injection were only observed in older adults.

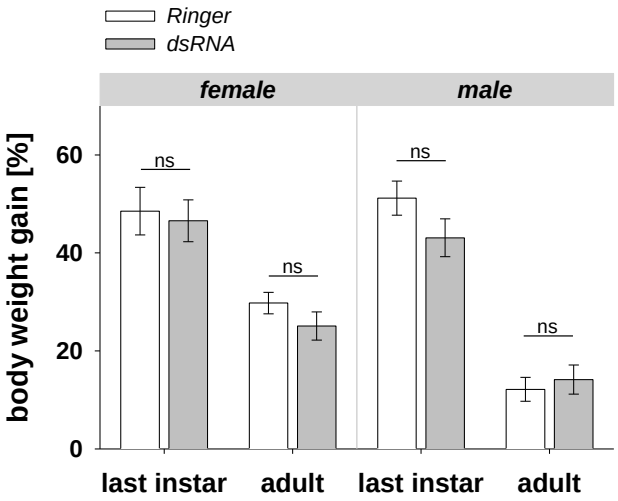

Figure 15: Weight gain of 2-day-old *G. bimaculatus* last instar larvae and adults, injected with either 2 µg AST-A dsRNA (in 10µl Ringer) or Ringer only at the day of the preceding moult. The body weight on the day of injection was set 100%. Mean ± SEM, n = 16-20. Statistics: students t-test, ns = not significant.

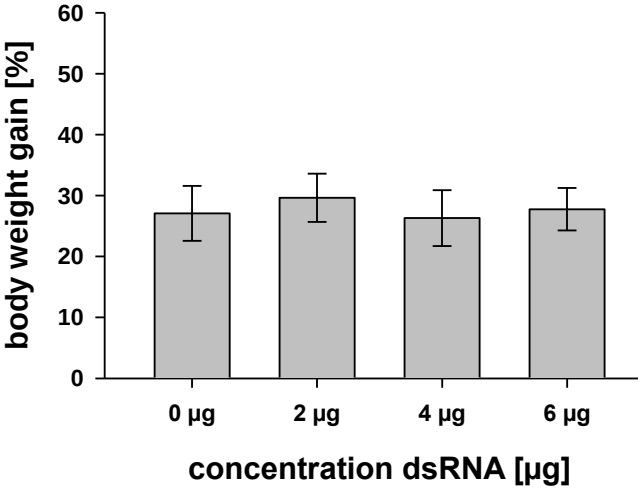

Figure 16: Weight gain of 2-day-old adult *G. bimaculatus* females, injected with AST-A dsRNA (0-6 µg in 10 µl Ringer) at the day of imaginal moult. The body weight on the day of injection was set to 100%. Mean ± SEM., n = 9–10. Statistics: ANOVA, P > 0.05 = no significant differences.

Newly moulted male and female *G. bimaculatus* adults and last instar larvae were injected with either 2 µg AST-A dsRNA or Ringer. Two days later, the enzyme activity of trypsin, aminopeptidase, lipase, and amylase was determined in the luminal content (Figure 17), tissue homogenate (Figure 18), and tissue incubation medium (Figure 19). There was no clear trend in enzyme activity of the luminal content for either last instar larvae or adult crickets following AST-A gene knockdown (Figure 17). The enzyme activity in the caecal lumen is an estimate of the amount of enzymes that have accumulated at a given age and time. Therefore, the experimental time of two days may have been too short to detect significant changes in enzyme activity in the lumen content after injection of 2 µg AST-A dsRNA.

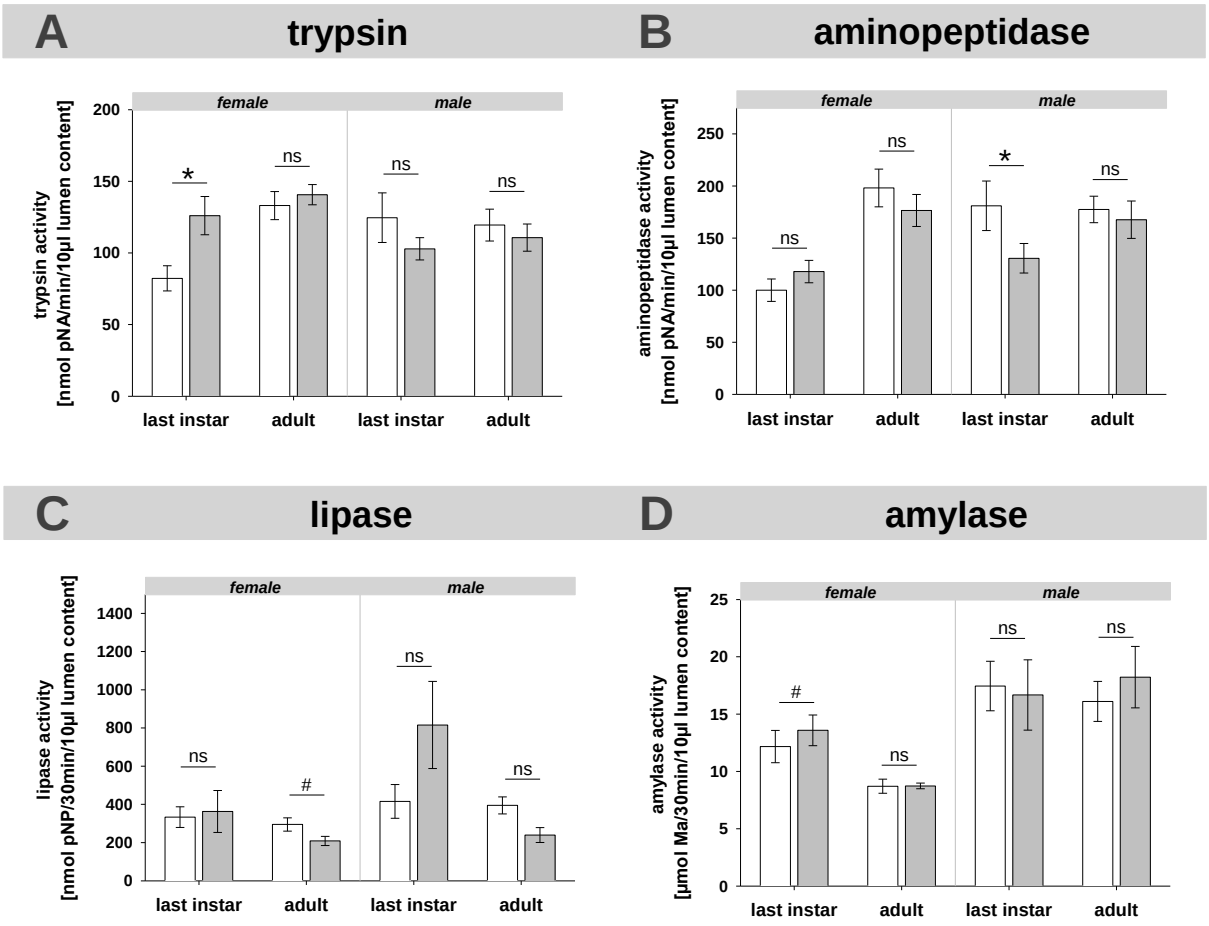

Figure 17: Activity of (A) trypsin, (B) aminopeptidase, (C) lipase and (D) amylase in the caecal lumen content of 2-day-old *G. bimaculatus* adults and last instar larvae injected with either 2 µg AST-A dsRNA (in 10 µl Ringer) (grey) or Ringer only (white) at preceding moult. Mean + SEM. n = 9-10. Statistics: Mann-Whitney U-test or student's t-test. ns = not significant, * = P < 0.05, # = 0.05 < P < 0.1.

However, there was a significant reduction of protease (Figure 18A,B), lipase (Figure 18C) and amylase activity (Figure 18D) in tissue homogenate of male and female crickets (with few exceptions) following AST-A gene knockdown. The enzyme activity in the tissue homogenate represents the amount of enzymes stored in tissue cells and, therefore, represents an indicator of enzyme synthesis rate. Thus, gene silencing of allatostatin type A reduced the synthesis of digestive enzymes in the midgut of *G. bimaculatus*.

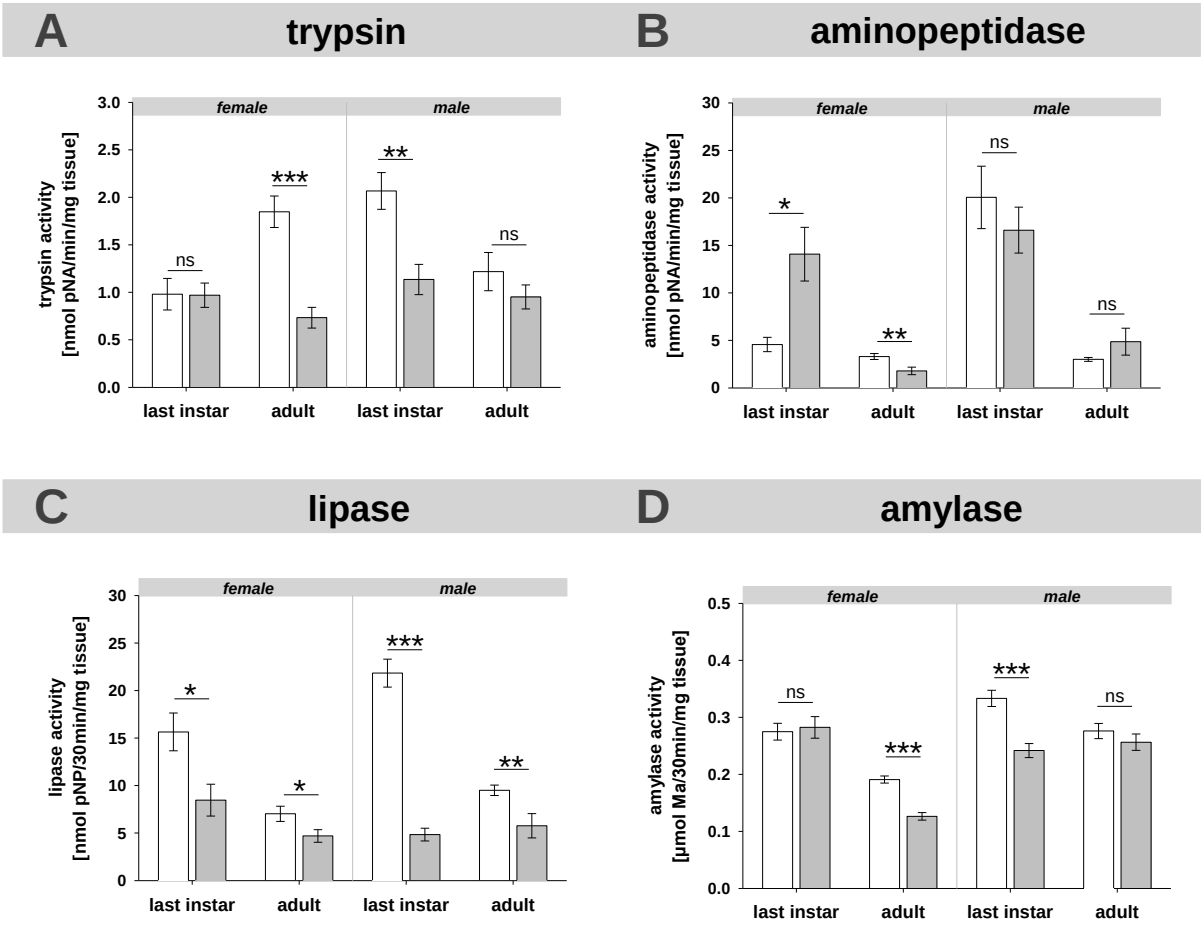

Figure 18: Activity of (A) trypsin, (B) aminopeptidase, (C) lipase and (D) amylase in the caecal tissue homogenate of 2-day-old *G. bimaculatus* adults and last instar larvae injected with either 2 µg AST-A dsRNA (in 10 µl Ringer) (grey) or Ringer only (white) at preceding moult. Mean + SEM. n = 9-10. Statistics: Mann-Whitney U-test or student's t-test. ns = not significant, * = P < 0.05, ** = P< 0.01, *** = P < 0.001.

Although, gene silencing of allatostatin A resulted in a reduced synthesis rate of digestive enzymes in the caecal tissue, there was a trend of increasing protease (Figure 19A,B) and lipase (Figure 19C) release from the caecal tissue into the incubation medium, especially in female last instar larvae.

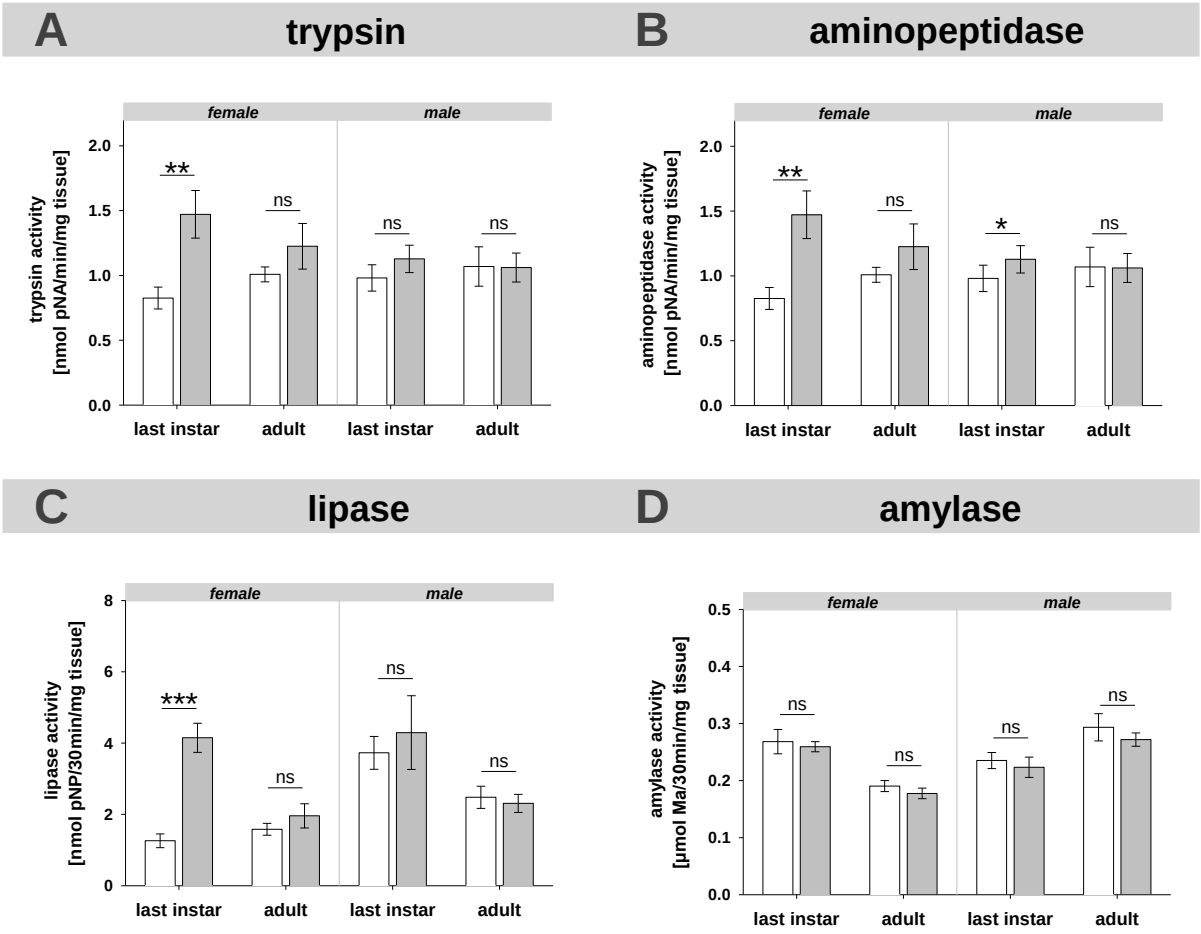

Figure 19: Activity of (A) trypsin, (B) aminopeptidase, (C) lipase and (D) amylase in the caecal tissue incubation medium of 2-day-old *G. bimaculatus* adults and last instar larvae injected with either 2 µg AST-A dsRNA (in 10 µl Ringer) (grey) or Ringer only (white) at preceding moult. Mean + SEM. n = 9-10. Statistics: Mann-Whitney U-test or student's t-test. ns = not significant, * = P < 0.05, ** = P< 0.01, *** = P < 0.001.

In general, larval and adult females of *G. bimaculatus* seem to be more sensitive to physiological effects on digestive enzymes caused by injections of AST-A dsRNA, than males. In the following experiment the effects of different concentrations of AST-A dsRNA injections on the digestive enzyme activities of adult females were analysed. In addition the concentration of soluble proteins was determined for each sample.

The amount of total soluble protein in samples of the luminal content (Figure 20C) decreased at higher amounts of AST-A dsRNA, but not in samples of tissue incubation medium or tissue homogenates (Figure 20A,B).

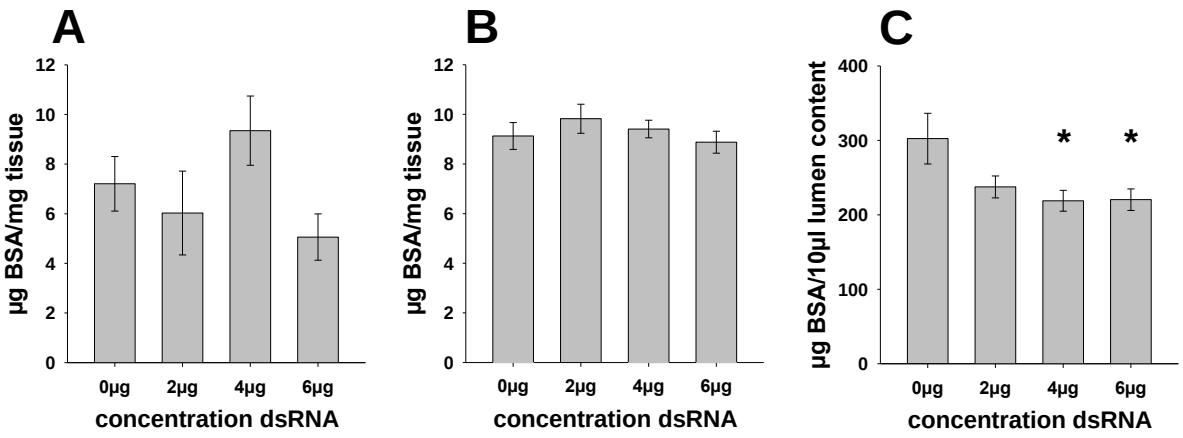

Figure 20: The effect of various concentrations of AST-A dsRNA on the protein concentration in (A) tissue incubate, (B) tissue homogenate, and (C) luminal content of 2-day-old adult *G. bimaculatus* females. The AST-A dsRNA (0-6 µg in 10 µl Ringer) was injected into crickets at day of imaginal moult. Mean ± SEM. n = 9-10. Statistics: ANOVA and post hoc Bonferroni t-test. * indicates significant differences to control (0 µg dsRNA).

The activities of amylase, aminopeptidase, trypsin, and lipase were analysed in luminal content, tissue homogenate and tissue incubation medium (secretion) (Figure 21). Injection of AST-A dsRNA did not show a dose-dependent effect on enzyme activity neither in tissue homogenate nor in tissue incubation medium, for all tested enzymes. However, higher concentration of 6 µg AST-A dsRNA resulted in significant higher amylase activity in the lumen content. Similar trends of increasing enzyme activity in the lumen content were observed for aminopeptidase and trypsin, respectively (Figure 21B,C).

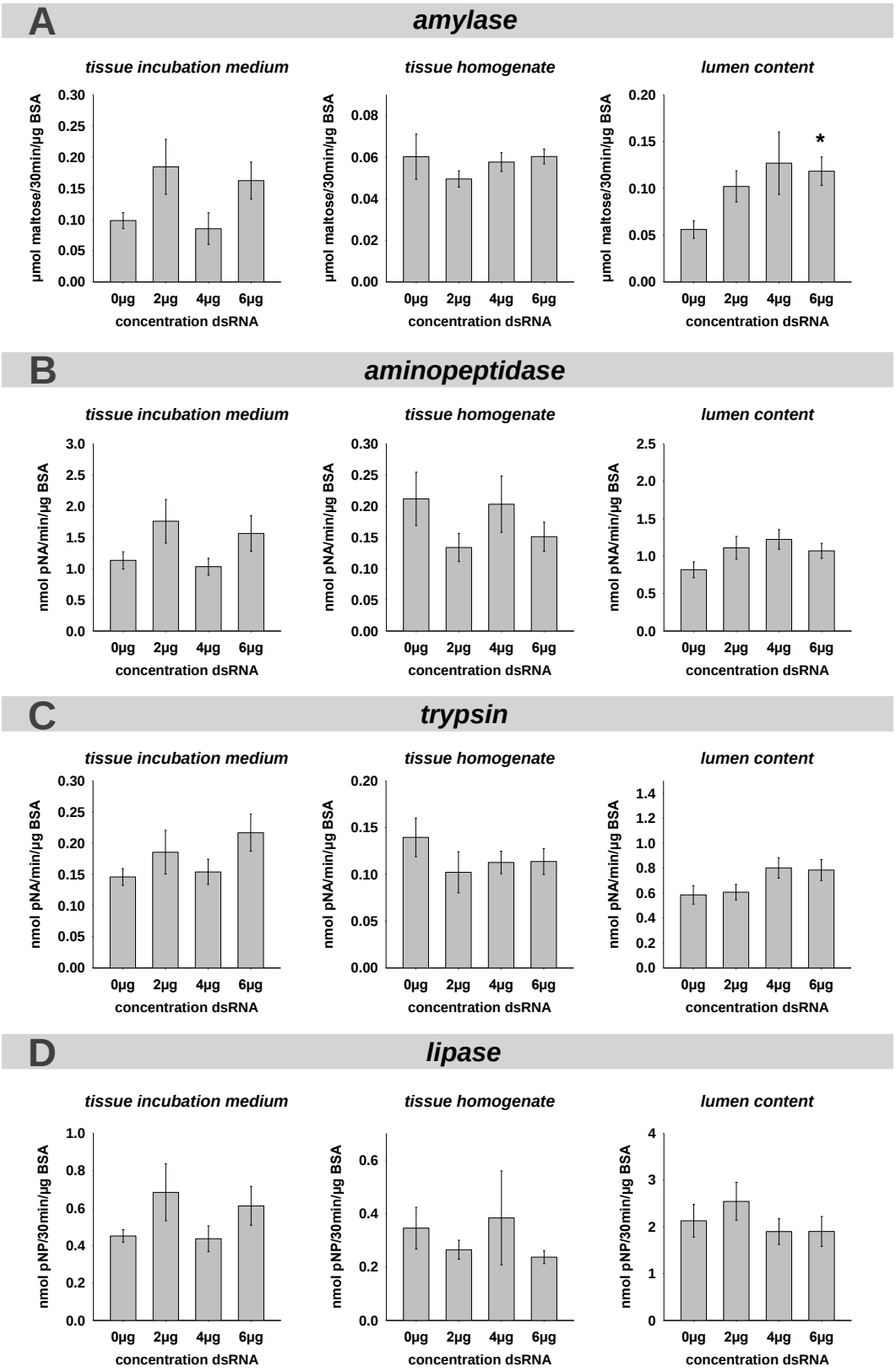

Figure 21: The effect of various concentrations of AST-A dsRNA on (A) amylase, (B) aminopeptidase, (C) trypsin, and (D) lipase activity in tissue incubation medium, tissue homogenate and lumen content of 2-day-old adult *G. bimaculatus* females. The AST-A dsRNA (0-6 µg in 10 µl Ringer) was injected at day of imaginal moult. Mean + SEM. n = 9-10. Statistics: Kruskal-Wallis test + post hoc Dunn's method. * indicates significant differences to control (0 µg dsRNA).

2.2.2.2 Sulfakinins

Previous studies using RNAi suggested that SK peptides affect satiety in *G. bimaculatus* by reducing food uptake (Meyering-Vos and Müller, 2007a), as was also reported for cockroaches and locusts (Wei et al., 2000; Maestro et al., 2001). A stimulating effect of sulfakinins on digestive enzyme release has already been demonstrated for beetles and moths (Nachman et al., 1997; Harshini et al., 2002b), but preliminary RNAi studies in *G. bimaculatus* showed no effect (Meyering-Vos and Müller, 2007a). Therefore, RNAi experiments regarding gene silencing of sulfakinin were repeatedly done, and subsequent physiological effects were analysed in more detail. The SK dsRNA was generated as previously described by Meyering-Vos and Müller (2007a). Male and female crickets were injected with either 2 µg dsRNA in 10 µl Ringer solution or 10 µl Ringer solution (control) with a 100 µl Hamilton syringe between the third and the fourth abdominal segment on the day of imaginal moult. Crickets were dissected two days after injection and all samples were analysed for soluble protein concentration and enzyme activities of amylase, cellulase, aminopeptidase, trypsin, and lipase.

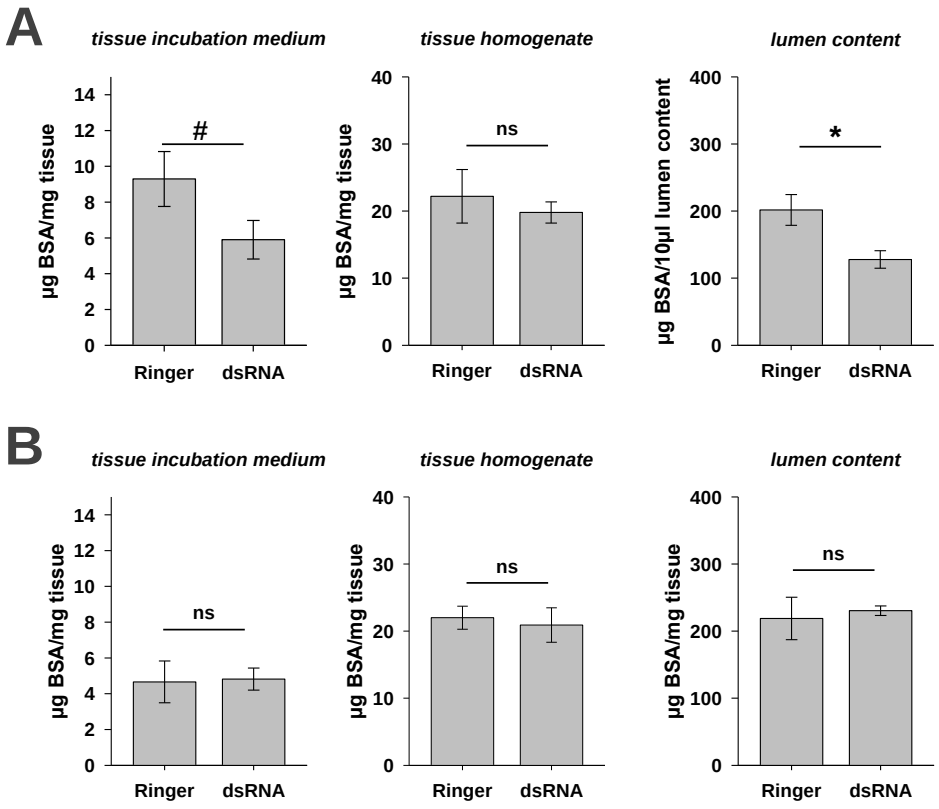

Figure 22: Protein concentration in samples of tissue incubation medium, tissue homogenate and lumen content from (A) female and (B) male 2-day-old adult *G. bimaculatus*, injected with either 10 µl Ringer (control) or 2 µg SK dsRNA in 10 µl Ringer at the day of imaginal moult. Mean ± SEM. n = 9-10. Statistics: student's t-test or Mann-Whitney U-test. * = P < 0.05, # = 0.05 < P< 0.1, ns = P> 0.1.

Injection of SK dsRNA induced a significant decrease in the protein concentration of lumen samples of females (Figure 22A). A similar trend was observed for the female tissue incubate. SK dsRNA injections had no effect on the protein concentrations in samples of male crickets (Figure 22B).

Gene silencing of sulfakinin resulted in higher amylase and cellulase release in female crickets (Figure 23A), but not in males. Therefore, SK peptides seem to have an inhibitory effect on carbohydrate digestion, at least in female *G. bimaculatus*.

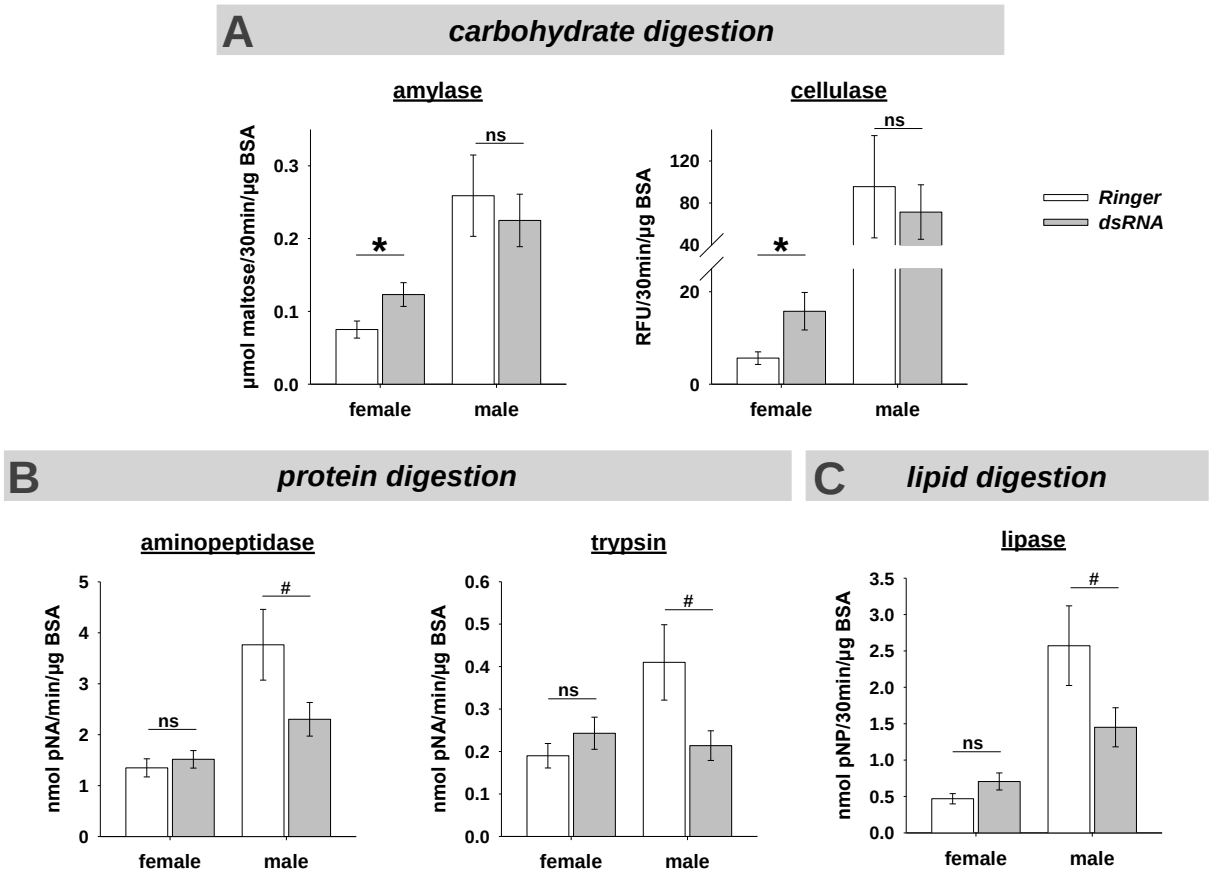

Figure 23: Effect of Ringer and SK dsRNA injection on enzyme secretion of (A) carbohydrases, (B) proteases and (C) lipase of 2-day-old adult female and male *G. bimaculatus*. 2 µg SK dsRNA in 10 µl Ringer or Ringer alone (control) was injected to crickets at the day of imaginal moult. Mean ± SEM. n = 9-10. Statistics: t-test or Mann-Whitney U-test. * = P < 0.05, # = 0.05 < P <0.1, ns = not significant.

Furthermore, there was a trend of decreasing protease (Figure 23B) and lipase (Figure 23C) release in male crickets, which may indicate a stimulatory effect of SK peptides on protein and lipid digestion. Injections of SK dsRNA did not affect enzyme activities in lumen content and tissue homogenate (not shown).

2.2.3 Calcium ions

In nerve cells, neuropeptides bind to receptors leading to a cascade of signals which regulate exocytosis of many kinds of proteins. In regulated exocytosis an external signal is required, resulting in the release of internal calcium stores. In the case of enzyme secretion calcium ions also play an important role in exocrine enzyme secretion.

The effect of calcium ions on the secretion of trypsin, amylase, cellulase and chitinase from the caecal epithelium was determined by comparison of the secretion rates in calcium free LGR and in the standard LGR (2 mM Ca^{2+}). Caecal tissue from single crickets was divided into half, and incubated in LGR either with or without calcium ions. The secretion rate of the incubate without calcium was set to 100% (control) and the effect of calcium ions on enzyme secretion was demonstrated as relative enzyme activity [%].

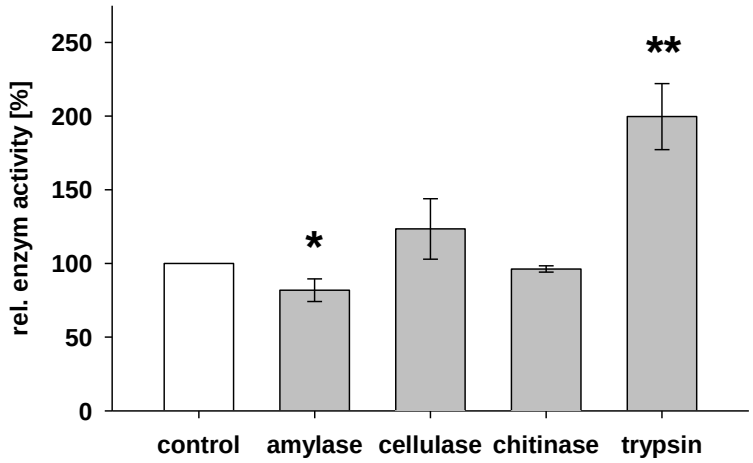

Figure 24: ***In vitro*** **effect of calcium ions (2 mM) on amylase, cellulase, chitinase, and trypsin secretion from caecal epithelium of 2-day-old adult female** ***G. bimaculatus*****.** Relative enzyme activity [%] compared to internal control (incubate without calcium). Mean ± SEM. n = 10-15. Statistics: paired t-test. Asterisks indicate significant differences to control:* = P <0.05, ** = P <0.01.

The addition of calcium ions to the incubation medium had little or no effect on cellulase and chitinase secretion from caecal tissue. Amylase secretion showed an inhibitory effect by calcium ions, with a decrease in activity of 25% (Figure 24), indicating a possible apocrine secretion mechanism as reported for larvae of *Tenebrio molitor* (Cristofoletti et al., 2001) (publication 2). In contrast, trypsin release was stimulated up to 100% by calcium ions compared to control, indicating a calcium-dependent exocytosis secretion mechanism (publication 4).

2.2.4 Trypsin activation and autolysis

In vertebrates, the protease trypsin is stored in cells as an inactive form (trypsinogen) before released and activated in the digestive tract. The presence of such trypsin precursors in insects has been rarely demonstrated and in only few species (Davis et al., 1985; Moffatt and Lehane, 1990; Barillas-Mury et al., 1991; Graf et al., 1991; Ramos et al., 1993). To investigate the presence or absence of a trypsin precursor in *G. bimaculatus*, samples of tissue homogenate incubated at 37°C for 4 h were analysed for its trypsin activity every 30 min (publication 4). The increasing trypsin activity at constant temperature within one hour indicates the presence of trypsin precursor stored in the secretory granules of the midgut endothelium of *G. bimaculatus* (Fig. 2, publication 4).

The molecular weight of the *Gryllus*-trypsin was calculated by SDS-PAGE (publication 1). Electrophoresis of samples from the luminal content was carried out under non-reducing conditions without sample heating in a 12% polyacrylamide mix gel with 0.1% SDS at a pH of 8.8 at room temperature. The SDS was removed and the proteins of different fractions were eluted from the gel and tested for trypsin activity. One fraction showed high trypsin activity and was further analysed by SDS-PAGE under reducing conditions. A calculated molecular weight of 23-24 kDa was indicated for *Gryllus*-trypsin from the luminal content (Fig. 2C, publication 1). After activation, *Gryllus*-trypsin showed no loss of activity within 4 h incubation at 37°C, while the activity of purified bovine trypsin decreased rapidly (Fig. 5, publication 4). It seems likely that the peptides resulting from protein digestion in the lumen (and possibly in combination with other ions) protect the trypsin from autolysis.

2.2.5 Endogenous protease inhibitors

The presence of endogenous protease inhibitors (EPI), which probably play a role in digestion regulation, has been demonstrated for several cockroach species (Elpidina et al., 2001; Vinokurov et al., 2007; Taranushenko et al., 2009). In order to investigate the presence of putative EPI in the midgut of *G. bimaculatus*, samples of tissue incubation medium, tissue homogenate and lumen content were divided in half. One half was heated at 90°C for 10 min to inactivate enzymes, the other half stayed untreated. The heated samples contained no active endogenous enzymes, but should contain active EPI as most Kunitz and Kazal type inhibitors are resistant to high temperatures. 50 µl Ringer (LGR in samples of tissue incubation medium) and 100 µl of the exogenous enzyme bovine trypsin (10 µg/100 µl) were added to 50 µl heated and unheated samples, respectively. Mild shaking at 30°C for 5 min should allow the EPI to bind to the exogenous enzyme. Afterwards, the trypsin activity in all samples and controls (Ringer solution + bovine trypsin) was determined for fed and starved crickets (Figure 25). The control was set to 100%.

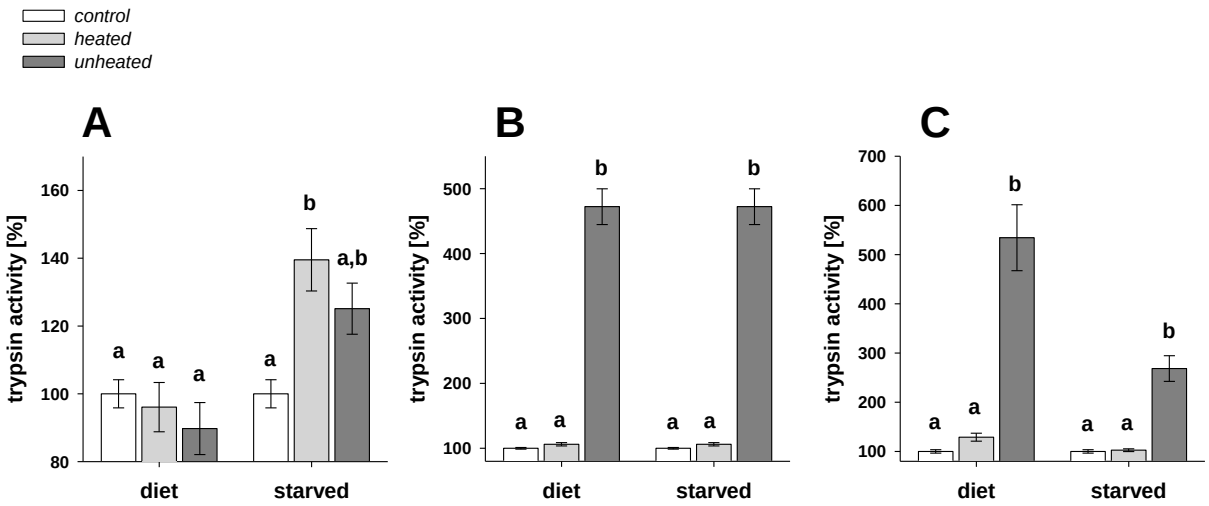

Figure 25: Trypsin activity in mixtures of exogenous bovine trypsin and heated and unheated samples of (A) tissue incubation medium, (B) tissue homogenate and (C) lumen content of diet-fed and starved 2-day-old adult *G. bimaculatus* females. Relative trypsin activity of the control (Ringer + bovine trypsin) was set to 100%. Heated samples contained inactive endogenous enzyme, unheated samples contained active endogenous enzymes. Bovine trypsin in equal concentration was added to all samples. Mean ± SEM. n = 10. Statistics: Kruskal-Wallis test and post hoc Tukey or Dunn's test. Different letters indicate significant difference between samples (control, heated, unheated).

There was no decrease of trypsin activity in heated samples compared to the controls for any tested samples (Figure 25). Therefore an inhibitory effect of a putative EPI in the midgut of *G. bimaculatus* could not be demonstrated.

2.3 The effect of environmental factors

Exogenous factors such as temperature, light-dark cycle, food quality and quantity affect the rate of digestive enzyme release and are closely linked to endogenous factors. Therefore, exogenous factors are important for the adaptation of an insect to its environment. Thus, the effects of incubation temperature and rearing temperature were analysed, particularly with regards to a putative acclimation effect. Furthermore, enzyme activity, enzyme release, and food uptake were documented within a 24 h light-dark cycle in penultimate instar larvae, last instar larvae and adult crickets. Food is one of the most important factors, affecting the secretion of enzymes into the digestive tract. Therefore, the effect of feeding and starvation, the influence of different nutrients and the effect of plant protease inhibitors on enzyme activity and secretion were tested.

2.3.1 Temperature

Temperature is one of the most important environmental factors that influences physiological processes in insects (Hoffmann, 1974; Merkel, 1977; Behrens et al., 1983; Haderspeck and Hoffmann, 1991). All processes (e.g. feeding rate, digestive enzyme activity, developmental time, egg production, metabolic rate, lethal temperature etc.) have an optimal temperature or temperature range, at which the activity is maximal, but not all activities have the same optimal temperature.

2.3.1.1 Incubation temperature

The digestive enzymes from the midgut of *G. bimaculatus* showed a broad optimal temperature range between 35°C and 40°C when animals were reared at 27°C (Table 1). Routinely, the amount of enzyme release was determined by incubation of the caecal tissue in LGR (see 2.1.3) at 37°C for 30 min. In order to determine the effect of incubation temperature on digestive enzyme release, caecal tissue of individual crickets were split in half and each half was incubated in LGR for 30 min at either 25°C or 35°C. Afterwards, the tissue was discarded, the incubate centrifuged and the supernatant was used for the enzyme assays.

Higher incubation temperature resulted in a positive effect on protease release in both male and female crickets (Figure 26A,B). In contrast, there was no temperature effect on the release of lipase (Figure 26C) and amylase (Figure 26D).

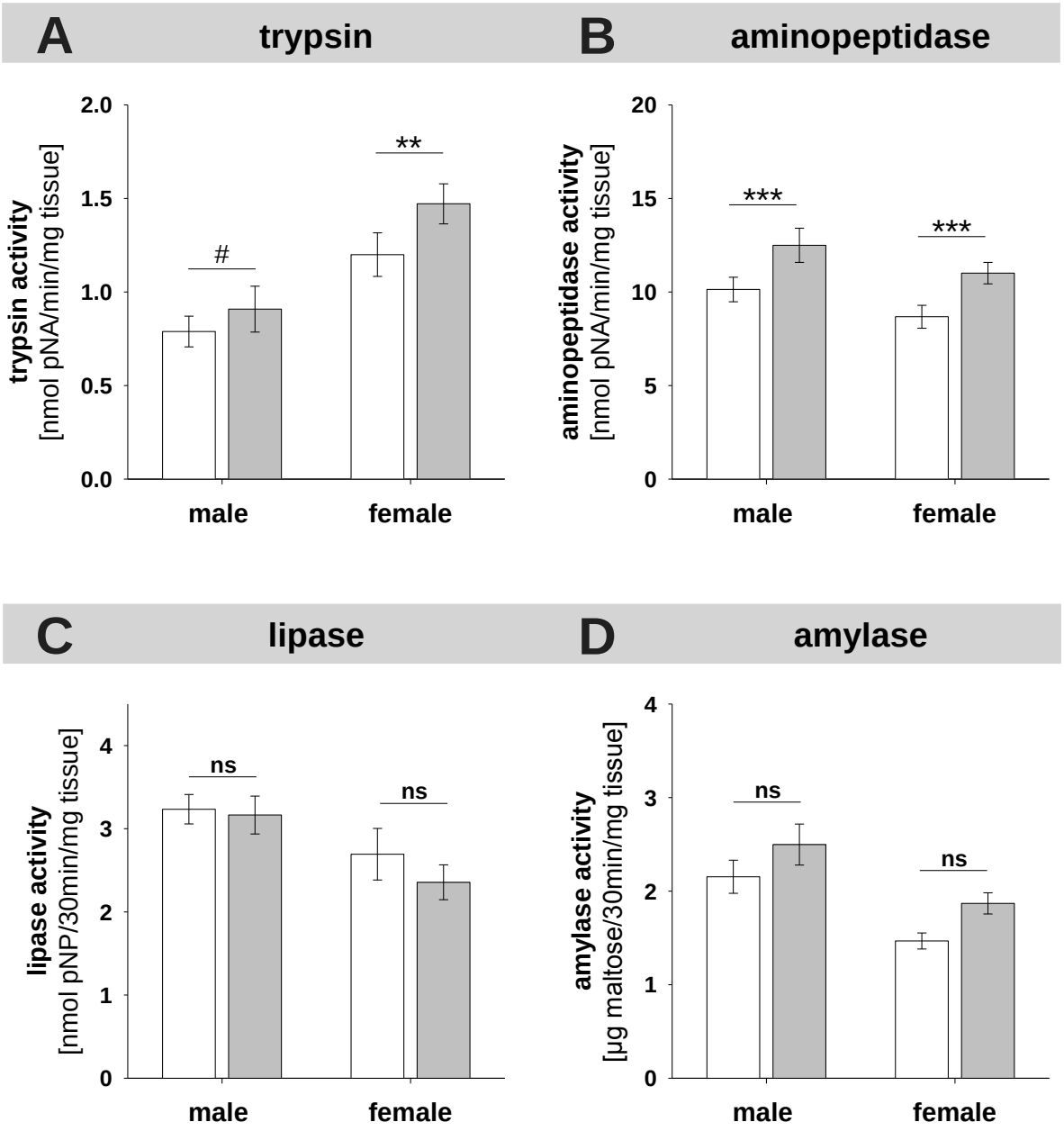

Figure 26: The effect of incubation temperature (25°C = white, 35°C = grey) on (A) trypsin, (B) aminopeptidase, (C) lipase, and (D) amylase secretion from caecal tissue of 2-day-old adult *G. bimaculatus*. Mean ± SEM. n = 19-35. Statistics: paired t-test or Wilcoxon test. ns = not significant (P > 0.05), # = 0.05 > P < 0.1, ** = P < 0.01, *** = P < 0.001.

2.3.1.2 Rearing temperature

Physiological processes in insects typically show either no temperature acclimation or a positive acclimation (higher rate at lower acclimation temperature). Acclimation can be either translational (constant Q_{10}) or rotational (changing Q_{10}) (Prosser, 1991). In order to investigate digestive enzyme release towards a possible temperature acclimation crickets were reared at either 22°C or 32°C from different times of development: (1) from imaginal moult, (2) from moult to last instar, (3) from moult to penultimate instar or (4) from egg deposition. Crickets

were dissected at day two of adult life and enzyme release was determined. As temperature has a profound effect on the developmental time, exposure time differed between the individual groups (1-4) and both acclimation temperatures (AT), and was greatly extended at 22°C compared to 32°C (Table 1, publication 4).

There was no temperature acclimation of lipase release, but trypsin showed a positive acclimation (Fig. 3C, publication 1) whereat trypsin secretion was always higher in the crickets acclimated to 22°C than those acclimated to 32°C.

2.3.2 Light-dark cycle

Photoperiod and the developmental stage of the crickets significantly affected the food uptake of *G. bimaculatus*, but also the secretion of digestive enzymes into the midgut. The secretion of carbohydrases (amylase and cellulase), proteases (aminopeptidase and trypsin) and lipase was examined in 2-day-old crickets of the penultimate larval instar, the last larval instar and the adult stage over the course of 24 h. Furthermore, the crop weight at the moment of dissection was determined as an indicator for feeding activity.

Crop weight in last instar larvae and adult crickets was highest at the beginning of the scotophase, but for unknown reasons not in the penultimate instar (Figure 27) (Fig. 11C, publication 2) (Fig. 5B, publication 3). The increase in food uptake may result from increasing locomotor activity, because last instar larvae and adult crickets are basically nocturnal (Woodring and Clifford, 1986; Matsui et al., 2009; Faßold et al., 2010).

The secretion of the two carbohydrases, amylase and cellulase, peaked in the photophase of 2-day-old larvae and adults of *G. bimaculatus* (Figure 27) (Fig. 11, publication 2). In contrast, protease secretion (aminopeptidase and trypsin) increased over time up to the scotophase (Figure 27) (Fig. 5B, publication 1). In last instar larvae and adult crickets lipase secretion increased up to the beginning of the scotophase (24:00 CEST), but in the penultimate larval instar lipase secretion peaked in the early photophase (8:00 CEST).

In general, the release of various digestive enzymes in crickets, reared under a long-day regime (LD 16:8 h photoperiod) seems to be regulated also by the light-dark change and does not correlate with feeding alone. This regulation mechanism may result from the influence of neuropeptides associated with digestive enzyme release or from the circadian regulation of locomotor activity (Matsui et al., 2009, 2013).

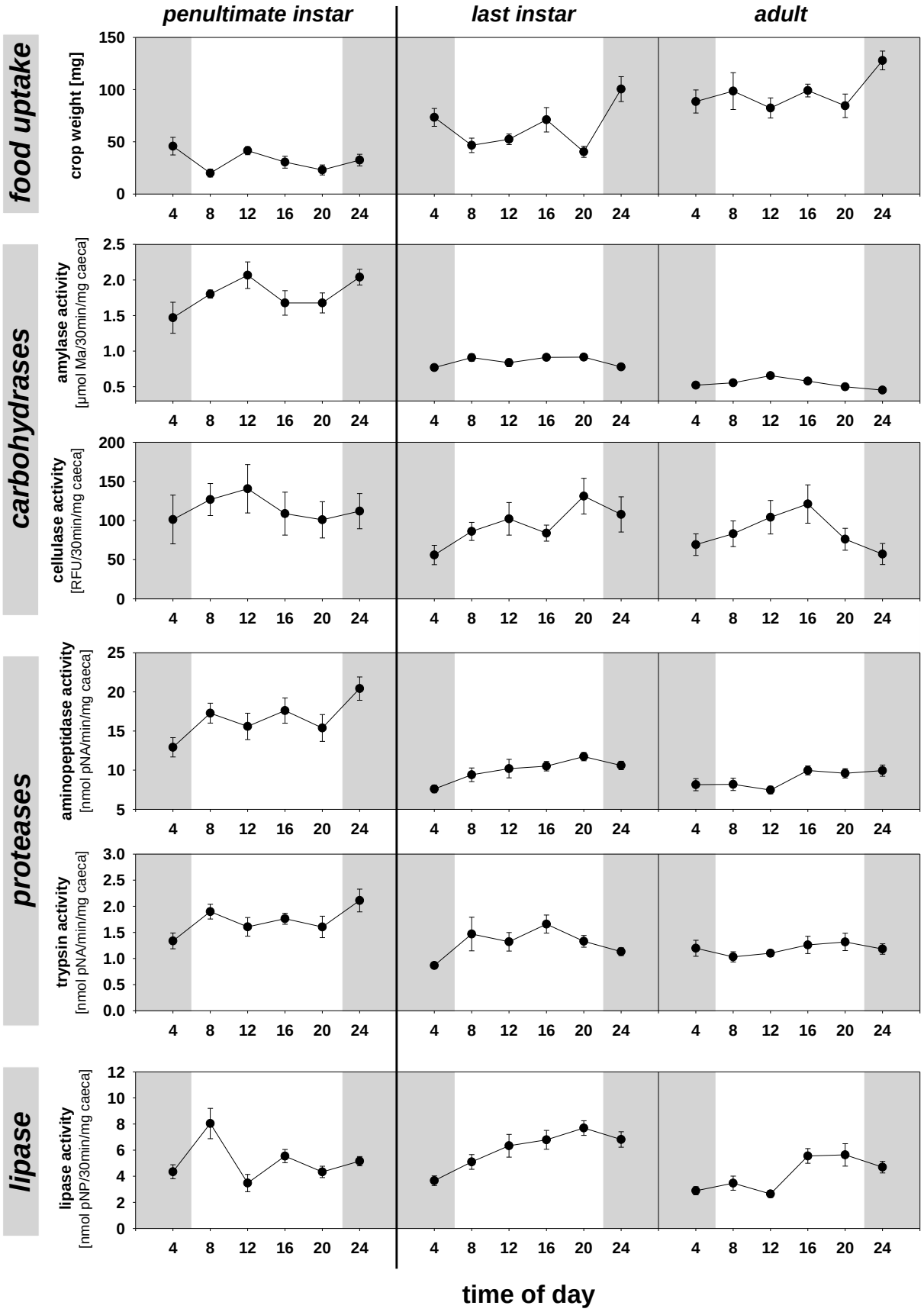

Figure 27: Food uptake and digestive enzyme secretion in the course of a day in 2-day-old penultimate larvae, last instar larvae and adult *G. bimaculatus* females. Scotophase was from 22:00-6:00 CEST (grey). Mean ± SEM. n= 9-10.

2.3.3 Food and nutrients

Feeding is the most important exogenous factor affecting enzyme secretion, which correlates with both the quality and the quantity of food. The importance of feeding on the secretion of digestive enzymes in *G. bimaculatus* was analysed in detail in publications 1 - 4.

The effect of food availability on enzyme secretion was determined in either 'feeding and starvation' or 'refeeding' experiments, while the importance of food quality was investigated by feeding different diets, and by *in vitro* incubation of caecal tissue in LGR in the presence of various nutrients.

2.3.3.1 Feeding and starvation

In order to test the effect of feeding and starvation on digestive enzyme release, crickets were placed individually into boxes shortly after imaginal moult to prevent cannibalism. They were provided with either a fresh cube of standard agar-diet or no food at all. Two days later the activities of amylase, cellulase, trypsin and lipase was determined in samples of tissue incubation medium, tissue homogenate and luminal content. For the refeeding experiment newly moulted crickets were isolated and not fed for 5 days. Afterwards, they were provided with the agar-standard diet and the release of amylase and chitinase was determined every hour.

The enzyme release and enzyme activity in midgut lumen for amylase (Fig. 4, publication 2), cellulase (Fig. 3, publication 2), trypsin (Fig. 6, publication 1), and lipase (Fig. 2, publication 3), strongly increased in the presence of food compared to starvation. Enzyme activities in tissue homogenate were also significantly higher in fed crickets compared to starved ones.

In refeeding experiments, amylase showed no response to the food uptake within 5 h (Fig. 5A, publication 3). However, 24 h later there was a significant increase of secretion indicating a strong decrease of amylase synthesis in the caecal tissue during starvation (Fig. 5B, publication 2). At least 6 h are required by caecal tissue to respond to the presence of food. In contrast, chitinase secretion was slightly increased shortly after food uptake (~ 10% in 2 h), but generally remained at low level (Fig. 6, publication 2).

In conclusion, feeding has a significant positive influence on digestive enzyme activity and stimulates not only the secretion of digestive enzymes, but also the synthesis rate in the caecal tissue.

2.3.3.2 Nutrients

The influence of different nutrients on digestive enzyme release has already been reported for trypsin, aminopeptidase and amylase (Woodring et al., 2009; Digali et al., 2010). Therefore, the effects of nutrients were analysed only for lipase (publication 3) and cellulase (publication 2).

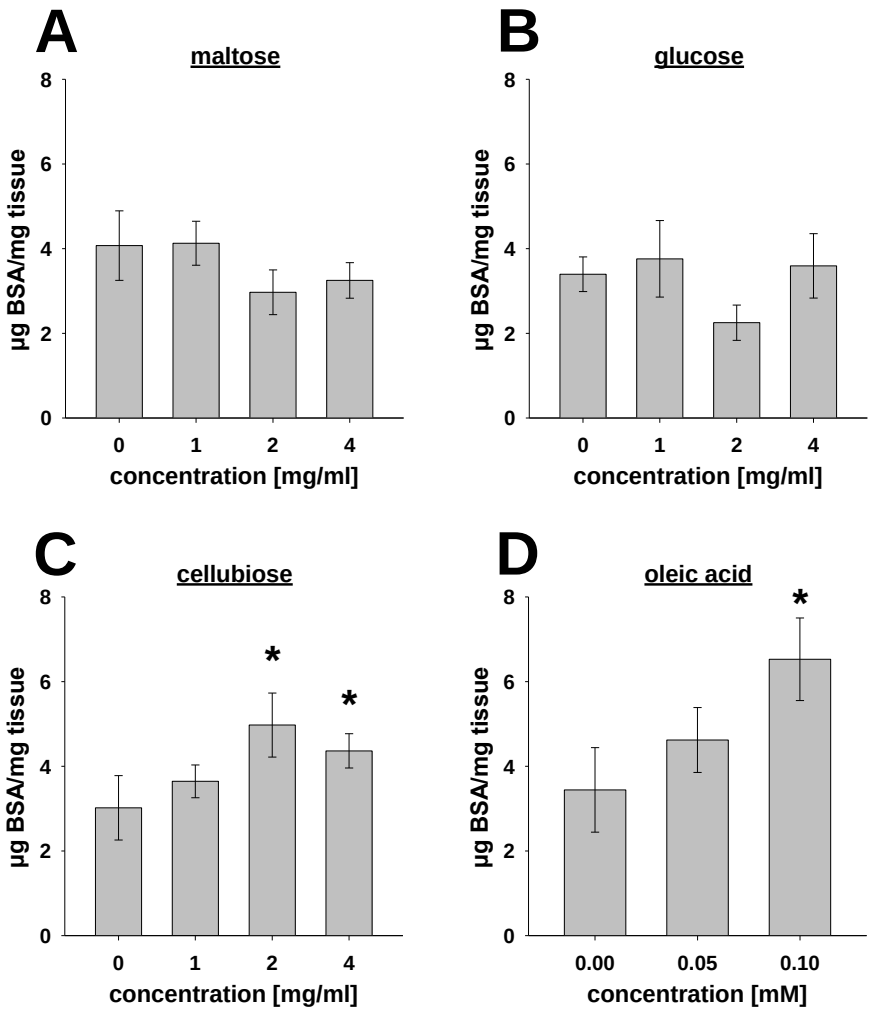

Figure 28: *In vitro* effects of nutrients on soluble protein concentration in the incubation medium of caecal tissue from 2-day-old adult female *G. bimaculatus*. Caecal tissues were incubated for 30 min at 37°C in LGR containing (A) maltose, (B) glucose, (C) cellubiose and (D) oleic acid. Mean ± SEM. n = 10. Statistics: Kruskal-Wallis test and post hoc Dunn's test with multiple comparisons versus control group (concentration = 0). * indicates significant differences to control group.

Additions of higher concentrations of cellubiose (2-4 mg/ml) and oleic acid (0.1 mM) to tissue incubation medium resulted in increased protein concentration and, therefore, in higher enzyme release from the caecal tissue (Figure 28C,D). There was no significant effect of maltose or glucose on protein concentrations (Figure 28A,B).

In vitro incubation of caecal tissue with oleic acid enriched LGR led to a significant higher lipase release from caecal endothelium (Fig 3, publication 3). Glucose and maltose had no effect on lipase activity (data not shown). In contrast to the stimulating effect of cellubiose on protein release from caecal tissue (Figure 28C), cellulase activity was much less in tissue incubation medium with increased concentration of cellubiose (Fig. 8A, publication 2). *In vivo* studies feeding cellulose-enriched diets (40, 70 or 100%) also resulted in decreased cellulase activity in luminal content and tissue incubation medium (Fig. 7, publication 2), and in reduced body weight (Fig. 9A, publication 2). However, the food uptake of the animals increased following feeding of 40-70% cellulose-enriched diets, which may indicate an attempt to compensate less energy uptake (Fig. 9B, publication 2).

2.3.3.3 Plant protease inhibitors

Crickets are omnivorous and may encounter plant material containing proteinase inhibitors, such as SBTI. Therefore, the adaptation of *G. bimaculatus* to SBTI enriched diet was studied in publication 1 in detail.

SBTI in the diet reduced trypsin activity in the lumen content and tissue incubation medium in a dose-dependent manner (Tables 1 and 2, publication 1). To investigate a putative adaptation of trypsin secretion to SBTI, newly moulted female last instar larvae were fed 0.1% and 0.4% SBTI enriched diets until day 2 after imaginal moult. *G. bimaculatus* seems to deal with lower concentrations of SBTI (0.1-0.2%) by hyperproduction of trypsin within the first 72 h, but are unable to adapt to higher concentrations (0.4%). Furthermore, larvae fed 0.4% SBTI enriched diet showed a reduced growth compared to crickets fed diet without SBTI (Fig. 9A, publication 1). Because plants are not the sole food source, *G. bimaculatus* is not under selective pressure to evolve a specific adaptation mechanism to protease inhibitors.

Summary

Insects are the most abundant animal species on earth with a huge economical and ecological impact. In spite of intensive research in the field of integrated pest management there are still a lot of questions concerning the adaptation mechanism of insects to their environment. As the digestive tract displays a putative target for effective pest management, this study worked on the effects of endogenous and environmental factors on digestive enzyme release in the omnivorous cricket, *Gryllus bimaculatus*.

The age-dependent enzyme release of carbohydrases, proteases and lipase correlates with the daily feeding rate of the crickets and peaked between days 2 to 4 in last instar larvae as well as in adult crickets. In contrast, the secretion of chitinase was affected by the moulting cycle of the insects reaching maximum activity at the day of moult. Therefore, chitinase plays only a minor role in food digestion. The cellulase activity in the midgut of *G. bimaculatus* resulted from an endogenous cellulase and was not caused by bacteria or eukaryotic endosymbionts in the digestive tract. The endoprotease trypsin was stored in the caecal tissue as an inactive precursor, and is secreted to the lumen by exocytosis. Following activation *Gryllus*-trypsin (~24 kDa) is protected from proteolytic degradation, but there is no endogenous protease inhibitor in the midgut.

Gene knockdown by RNA interference was used to analyse the endogenous regulation of digestive enzyme release by the neuropeptides allatostatin A and sulfakinin, which had already been shown to affect feeding in *G. bimaculatus*. Functional analysis of the AST-A gene was investigated for last instar larvae and adult crickets, whereby female crickets seemed to be more sensitive to this method. The gene suppression of AST-A resulted in a decreased synthesis of amylase, trypsin, aminopeptidase and lipase in the caecal tissue, but enzyme release varied between sexes and developmental stages. The knockdown of SK expression led to an increase of amylase and cellulase secretion in female crickets, and to a reduction of protease and lipase release in males.

As food plays a fundamental role in digestive enzyme release, both quality and quantity of nutrition are ample factors. There was always a higher digestive enzyme activity in fed crickets compared to starved ones. Furthermore, starvation resulted in a decrease of enzyme synthesis in the caecal tissue. In general, nutrients in the incubation medium led to a stimulation of digestive enzyme secretion, but in the case of cellulase the presence of both cellubiose in the incubation medium and cellulose in the diet caused a strong decline in cellulase release. Addition of the plant protease inhibitor SBTI to the diet caused a dose-dependent inhibition of protease activity in the caeca, whereby minor concentrations of SBTI were compensated by enzyme hyperproduction.

In addition to the food uptake, the daily light-dark cycle seems to affect digestive enzyme release. Crickets started to feed at the beginning of the scotophase, which led to an increase of protease and lipase secretion in larvae and adults. The secretion of carbohydrases was highest during the photophase. This means that enzyme release is not solely affected by the time of food uptake.

Temperature is one of the most important environmental factors, but seems to play only a minor role in the release of digestive enzymes. All tested enzymes showed a broad optimal temperature range (30°C - 40°C), but there was no difference in the release of amylase or lipase after tissue incubation at 25°C or 35°C. In contrast, trypsin and aminopeptidase showed a higher secretion after incubation at 35°C compared to 25°C. Furthermore, insect rearing at 22°C and 32°C during various developmental stages resulted in a positive acclimation of trypsin secretion to rearing temperature.

Zusammenfassung

Insekten stellen mit Abstand die größte und vielfältigste Tiergruppe auf Erden dar und üben einen großen ökologischen wie auch ökonomischen Einfluss aus. Trotz intensiver Forschung im Bereich der integrierten Schädlingsbekämpfung sind bislang noch viele Fragen über die Adaptionsmechanismen von Insekten gegenüber ihrer Umwelt ungeklärt. Da der Verdauungstrakt von Insekten ein potentielles Angriffsziel für effektive Schädlingsbekämpfung darstellt, wurde innerhalb dieser Studie die Freisetzung von Verdauungsenzymen bei der omnivoren Grille, *Gryllus bimaculatus*, in Abhängigkeit von endogenen und Umweltfaktoren (Temperatur, Licht-Dunkel Rhythmus, Futter) analysiert.

Die Freisetzung von Carbohydrasen, Proteasen und Lipasen in den Caeca von adulten und larvalen *G. bimaculatus* korreliert stark mit der täglichen Futteraufnahme. Dabei wurde eine maximale Enzymsekretion zwischen Tag 2 und 4 des letzten Larvenstadiums bzw. des Adultstadiums gefunden. Die Freisetzung von Chitinase wird hingegen maßgeblich vom Zeitpunkt der Häutung beeinflusst, so dass Chitinase bei der Nahrungsverwertung eher eine untergeordnete Rolle spielt. Darüber hinaus wird vermutet, dass die gemessene Cellulaseaktivität in den Caeca auf eine endogene Cellulase zurückzuführen ist, da aufgrund der Probenaufarbeitung mögliche eukaryotischen Endosymbionten oder Bakterien aus dem Darm entfernt wurden. Nähere Untersuchungen an der Protease Trypsin zeigten, dass das Enzym in Form einer Vorstufe (Zymogen) im Darmgewebe gespeichert wird. Die Freisetzung erfolgt über Exocytose aus den zymogenen Zellen des Mitteldarmes in das Darmlumen. Nach Aktivierung der Vorstufe wies das *Gryllus*-Trypsin ein Molekulargewicht von ~24 kDa auf und war gegen proteolytischen Abbau im Lumen geschützt. Eine Regulation der Proteaseaktivität im Mitteldarm durch endogene Protease-Inhibitoren konnte nicht gefunden werden.

Mithilfe der RNA-Interferenz Methode (Gen-Knockdown) wurde die endogene Steuerung der Enzymsekretion im Mitteldarm durch die Neuropeptide Allatostatin A und Sulfakinin näher untersucht. Beide Peptide beeinflussen nachweislich die Futteraufnahme der Tiere. Die Funktionsanalyse des Allatostatin A Gens auf physiologischer Ebene wurde an Larven und adulten Grillen durchgeführt, wobei Weibchen auf die Methode sensibler zu reagieren scheinen. Gensuppression von AST-A führte meist zu einer reduzierten Syntheserate von Amylase, Trypsin, Aminopeptidase und Lipase in den Caeca-Geweben. Dabei schwankte die Höhe der Freisetzung zwischen den untersuchten Stadien und Geschlechtern. Der Knockdown der Sulfakininexpression führte hingegen zu einer deutlichen Erhöhung der Amylase- und Cellulasesekretion bei Weibchen, während bei Männchen ein Trend zu reduzierter Protease- und Lipasefreisetzung ersichtlich war.

Sowohl die Quantität als auch die Qualität der Nahrung haben einen maßgeblichen Einfluss auf die Freisetzung aller untersuchten Enzyme. Gefütterte Tiere wiesen stets eine höhere Aktivität und eine stärkere Freisetzung der Verdauungsenzyme auf als gehungerte Grillen. Längere Hungerphasenführten zu einer Reduktion der Enzymaktivität im Darm und zu einer deutlich verminderten Syntheserate in den Caeca-Geweben. Obwohl die Anwesenheit von Nährstoffen im Inkubationsmedium eher stimulierend auf die Freisetzung der Enzyme wirkte, konnte bei Cellulase eine starke Reduktion der Sekretion bei Anwesenheit von Cellubiose im Inkubationsmedium oder Cellulose in der Nahrung festgestellt werden. Zusatz des pflanzlichen Protease-Inhibitors SBTI zur Nahrung führte zu einer konzentrationsabhängigen Reduktion der Aktivität und Freisetzung von Trypsin in den Caeca, wobei die Grillen geringere Konzentrationen an SBTI durch Enzym-Hyperproduktion kompensieren konnten.

Neben der Futteraufnahme wurde die Freisetzung der Verdauungsenzyme durch den Tag-Nacht-Rhythmus der Tiere beeinflusst. Das Einsetzen der Futteraufnahme zu Beginn der Dunkelphase erklärt die erhöhte Protease- und Lipasefreisetzung, jedoch konnte in adulten wie larvalen *G. bimaculatus* ein Anstieg der Freisetzung von Carbohydrasen während der Photophase verzeichnet werden. Die Regulation der Enzymfreisetzung wird folglich nicht allein durch den Zeitpunkt der Futteraufnahme bestimmt.

Temperatur als Umweltfaktor spielt bei der Freisetzung von Verdauungsenzymen in *G. bimaculatus* eine eher untergeordnete Rolle. Obwohl alle untersuchten Enzyme Temperaturoptima im Bereich von 30°C - 40°C aufwiesen, zeigten Lipase und Amylase keinen Unterschied in der Freisetzung nach Gewebeinkubationen bei 25°C oder 35°C. Die Sekretion von Proteasen hingegen war bei 35°C Inkubationstemperaturdeutlich erhöht. Bei längerer Zucht der Tiere bei 22°C und 32°C konnte nur für Trypsin eine Anpassung der Sekretionsrate an die Haltungstemperatur in Form einer positiven Akklimatisation gefunden werden.

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Part II

Publications and Manuscripts

Publications

Publications and manuscripts for dissertation:

Parts of this dissertation have already been published in peer-reviewed scientific journals:

Weidlich S., Müller S., Hoffmann K.H., Woodring J., 2013. Regulation of amylase, cellulase and chitinase secretion in the digestive tract of the two-spotted field cricket, *Gryllus bimaculatus.* Archives of Insect Biochemistry and Physiology 83: 69-75.

Weidlich S., Huster J., Hoffmann K.H., Woodring J., 2012. Environmental control of trypsin secretion in the midgut of the two-spotted field cricket, *Gryllus bimaculatus*. Journal of Insect Physiology 58: 1477–1484.

submitted manuscript:

Weidlich S., Hoffmann K.H., Woodring J., 2013. The secretion of lipase in the midgut of *Gryllus bimaculatus*: regulation by endogenous and environmental factors. Physiological Entomology.

manuscript will be submitted shortly

Weidlich S., Hoffmann K.H., Woodring J., 2013. Activation and autolysis of trypsin in the midgut of the Mediterranean field cricket, *Gryllus bimaculatus*.

Further publications

Lwalaba D., **Weidlich S.**, Hoffmann K.H., Woodring J., 2010. Exogenous and endogenous protease inhibitors in the gut of the fall armyworm larvae, *Spodoptera frugiperda*. Archives of Insect Biochemistry and Physiology 74: 114–126.

Author's contribution

publication 1

Weidlich S., Huster J., Hoffmann K.H., Woodring J., 2012. Environmental control of trypsin secretion in the midgut of the two-spotted field cricket, *Gryllus bimaculatus*.

The experiments were created and organized by myself. I carried out the main part of the experiments: kinetic parameters, identification of Gryllus-trypsin by SDS-PAGE, temperature acclimation, SBTI diets. Juliane Huster prepared samples and data for age-dependent trypsin secretion under my supervision. Prof. Joseph Woodring provided data on light-dark rhythm of trypsin secretion and the effect of feeding and starvation. Finally, I analysed and evaluated all data statistically, and wrote the manuscript. Own contribution: 90%

publication 2

Weidlich S., Müller S., Hoffmann K.H., Woodring J., 2013. Regulation of amylase, cellulase and chitinase secretion in the digestive tract of the two-spotted field cricket, *Gryllus bimaculatus.*

The major part of all experiments were created, organized and carried out by myself. Data on chitinase secretion were provided by Mario Schwartz, data on in vitro effect of nutrients on enzyme secretion was done by Sonja Müller. Finally, I analysed and evaluated all data statistically, and wrote the manuscript. Own contribution: 80%

publication 3

Weidlich S., Hoffmann K.H., Woodring J., 2013. The secretion of lipase in the midgut of *Gryllus bimaculatus*: regulation by endogenous and environmental factors.

I performed all experiments by myself, analysed and evaluated the data statistically, and wrote the manuscript. Own contribution: 99%.

publication 4

Weidlich S., Hoffmann K.H., Woodring J., 2013. Activation and autolysis of trypsin in the midgut of the Mediterranean field cricket, *Gryllus bimaculatus*.

I designed and organized the main part of the experiments. The experiments were carried out by Jörn Herfert and me. I analysed and evaluated all data, and wrote the manuscript. Own contribution: 75%.

Publication 1

Weidlich S., Huster J., Hoffmann K.H. and Woodring J.

Environmental control of trypsin secretion in the midgut of the two-spotted field cricket, *Gryllus bimaculatus*.

Journal of Insect Physiology (2012) 58:1477-1484.

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Publication 2

Weidlich S., Müller S., Hoffmann K.H. and Woodring J.

Regulation of amylase, cellulase and chitinase secretion in the digestive tract of the two-spotted field cricket, *Gryllus bimaculatus.*

Archives of Insect Biochemistry and Physiology (2013) 83: 69-75.

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Publication 3

Weidlich S., Hoffmann K.H. and Woodring J.

The secretion of lipase in the midgut of *Gryllus bimaculatus*: regulation by endogenous and environmental factors.

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The secretion of digestive lipase in the midgut of *Gryllus bimaculatus*: regulation by endogenous and environmental factors.

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Abstract. Lipase release in *Gryllus bimaculatus* depends on various endogenous (age, sex, developmental stage) and exogenous (light-dark cycle, food quality, temperature) factors. Whereas lipase secretion was very similar in both sexes of last instar larvae, lipase release peaked on day 5 after ecdysis in adult females and on day 7 in adult males; increased feeding resulted in increased lipase release. In last instar larvae and adults feeding and lipase release showed a circadian rhythm and increased from 4:00 to 24:00 CEST, but not in penultimate larvae. Lipase activity in the luminal contents and in caecal secretion was higher in fed crickets than in those fed a non-nutritive cellulose diet or starved. Increasing concentration of fatty acids in the caecal incubation medium led to increasing lipase release. The lipase release from caeca incubated at either 25°C or 35°C showed little difference. Crickets acclimated at 32°C showed higher lipase release than those acclimated at 22°C, indicating no temperature acclimation. Lipase secretion increased with a longer exposure time (adult stage, last larval stage, last two larval instars) when the crickets were acclimated at 32°C, but not when acclimated at 22°C. There was no difference in total food uptake in crickets maintained at 22°C or 32°C, but at 22°C development was slowed and, therefore, there was a longer time in which the daily food uptake was reduced.

Key words: lipase, digestive enzyme, cricket, temperature, food uptake, photoperiod

Introduction

The two-spotted field cricket, *Gryllus bimaculatus,* is an omnivorous insect adapted to consumption of plant, fungi and insect material. The secretion of digestive enzymes requires control mechanisms to cope with the variable quality and quantity of food found in nature. A regulation of enzyme secretion and synthesis is therefore essential (Blakemore *et al.*, 1995). The midgut is the main site of digestive enzyme release and metabolite absorption in insects (Dow, 1992; Chapman, 1998), whereby the regulation of digestive enzyme secretion is subject to various mechanisms (Lehane *et al.*, 1995). The midgut epithelium of insects consists of a single cell layer with numerous interspersed endocrine cells (Montuenge *et al.*, 1989; Endo *et al.*, 1990; Sehnal & Zitnan, 1996), which are likely to play a role in intestinal activities (Lehane *et al.*, 1996).

Numerous studies already demonstrated the action of various neuropeptides on digestive enzyme release in insects (Fusé *et al.*, 1999; Harshini *et al.*, 2002a, b; Aguilar *et al.*, 2003; Hill & Orchard, 2005; Sakai *et al.*, 2006; Woodring *et al.*, 2009; Lwalaba *et al.*, 2010). The release of neuropeptides is induced by nutrients in the lumen, and these peptides act as parahormones inducing the release of digestive enzymes from nearby zymogen cells in the endothelium (Lehane *et al.*, 1996). Therefore, the secretion of digestive enzymes is strongly correlated to the food intake of an insect (Engelmann, 1969; Dadd, 1970; Chapman, 1985; Terra, 1990; Lehane *et al.*, 1996; Terra *et al.*, 1996).

Lipids are utilized for energy storage (fat body) and for oogenesis in all insects, and in some insects (seed feeders) lipids are an important source of energy. For most insects, however, including crickets, very little dietary lipid is required for growth and development(Patton, 1967; Chippendale, 1971; Woodring *et al.*, 1979**),** but almost all insects have a specific dietary requirement for sterols and polyunsaturated fatty acids (Canavoso *et al.*, 2001). Many insects can obtain the essential polyunsatured fatty acids by digestion of phospholipids via secretion of phospholipase A_2 from the midgut endothelium (Rana & Stanley, 1999). Crickets have a very lipid rich fat body (over 50% triglycerides) (Lorenz & Gäde, 2009), but these lipids are primarily derived from ingested carbohydrates. Crickets, however do synthesize and release significant amounts of lipases into the midgut (Teo & Woodring, 1988; Woodring *et al.*, 2009), meaning that they can use nutrient lipids (triglycerides) for energy stores, but they are not required.

Complete lipid digestion is accomplished by carboxylic ester hydrolases (EC 3.1.1; lipases, esterases, phospholipase A and B), phosphoric monoester hydrolases (EC 3.1.3; phosphatases) and phosphoric diester hydrolases (EC 3.1.4; phospholipase C and D) (Terra *et al.*, 1996). Thereby, lipases (EC 3.1.1.3) are essential compounds of the fat metabolism and hydrolyse the outer ester links of triacylglycerols from the α-position stepwise to diacylglycerols, monoacylglycerols, glycerol and free fatty acids (Bollade *et al.*, 1970; Hoffman & Downer, 1979; Secundo *et al.*, 2006).

Lipid metabolic activities in the tissues of insects are well characterized (Canavoso *et al.*, 2001; Arrese *et al.*, 2001; Van der Horst & Ryan, 2012), but to date there are only a few reports on digestive lipases

from relatively few species., and secretion from gut tissue is not well understood (Weintraub & Tietz, 1973; Male & Storey, 1981; Mrdaković *et al.*, 2008; Horne *et al.*, 2009; Woodring *et al.*, 2009; Lwalaba *et al.*, 2010; Christeller *et al.*, 2010, 2011; Zibaee, 2012; Zibaee & Fazeli-Dinan, 2012). The current study focuses on the effect of extrinsic factors (temperature, light-dark cycle, food consumption) and intrinsic factors (age, developmental stage, sex) on the release of digestive lipase in the midgut of *G. bimaculatus*.

Materials and methods

Rearing method and feeding

The Mediterranean field cricket *G. bimaculatus* de Geer (Ensifera, Gryllidae) was raised under a long-day regime (LD 16:8 photocycle, light from 6 a.m. to 10 p.m. CEST) at 27°. Newly emerged crickets were isolated within 1 h after the imaginal moult and were designated 0-day-old crickets. Crickets received a mixed diet (cricket chow) consisting of ground rabbit, rat and cat food in a ratio of 4:2:1 (w/w), all from Altromin Lage, Germany. The total nutrient value of the cricket chow was 40 % carbohydrates, 25 % protein, and 6 % lipids (Lorenz & Anand, 2004).

The *in vivo* effect of feeding and starvation on enzyme release was tested by providing standard agar diet (40 g cricket chow + 3.6 g agar + 160 ml H_2O) and a non-nutrient cellulose diet (120 g cellulose powder + 12 g agar per litre H_2O) or by starvation (access to water, but no food at all). After imaginal moult crickets were placed individually into boxes and provided a fresh cube of an agar-diet or no food at all.

To investigate the influence of temperature acclimation on lipase release crickets were divided into two groups, one maintained at 22°C the other at 32°C (acclimation temperature, AT). Each of these two groups was divided into three groups, which were set to AT from (a) day 0 of the penultimate instar, (b) day 0 of the last instar, and from (c) day 0 of the adult life. Hence the crickets were exposed to the two AT for a short, intermediate or long period of time. During this time crickets were kept individually in boxes (10x10x6 cm). For each of the total of six groups the caeca were removed on day 2 of the adult stage, divided in half and each half incubated in low glucose Ringer at either 25°C or 35°C incubation temperature (IT) for 30 min. The results are given per mg caeca to compensate for the different size of each half. In addition, food uptake was determined for crickets acclimated at both 22°C and 32°C from the beginning of last larval stage.

Gut dissection and sample preparation

The crickets were ventrally cut open from the last abdominal segment to the neck. The caeca were removed, cut open and rinsed 3-times with *Gryllus* Ringer (138 mM NaCl, 5 mM KCl, 2 mM $CaCl_2 \cdot 2\ H_2O$, 4 mM Hepes, pH 7.2). Contraction of the muscles of the opened caeca leads to a cup-shaped structure with the lumen side outermost and the hemolymph side innermost, which was designated a flat-sheet gut preparation (Blakemore *et al.*, 1995).

To determine the lipase release (secretion), the opened and rinsed caecal tissue of individual crickets were incubated in low glucose Ringer (LGR) (10 mg glucose / 100 ml *Gryllus* Ringer) for 30 min at 37°C without shaking. The air-filled trachea kept the caeca at the surface of the medium. Following incubation, the caecal tissue was discarded and cells were removed by centrifugation at low speed (2000 g) for 2 min at 4°C.

To determine lipase activity in tissue cells, rinsed caecal tissue of individual cricket was added to 150 - 200 µl *Gryllus* Ringer with a few crystals of N-phenylthiourea (PTH) and homogenized with an ultrasonicator at the lowest setting for few seconds (Sonifier 250, Branson). Tissue homogenate (TH) was centrifuged at 16000 g for 10 min at 4°C and the supernatant was used for the enzyme assay.
To test the lipase activity in caecal lumen content, 10 µl aliquots of luminal contents were mixed with 190 µl *Gryllus*-Ringer with PTH and centrifuged at 16000 g at 4°C for 2 min.

Enzyme assay

Lipase activity was measured using the substrate *p*-nitrophenyl palmitat (*p*NPP) (Winkler & Stuckman, 1979). 15 mg *p*NPP was dissolved in 5 ml 2-propanol by heating to about 50°C until clear (8 mM *p*NPP stock solution). The stock solution was diluted with 50 mM Tris-HCl buffer, pH 8 + 0.1 % Triton X100 and gently mixed or heated until a clear suspension resulted (0.4 mM working solution). The Triton X prevented the formation of a turbid suspension (Gupta *et al.*, 2002).
190 µl of a freshly prepared 0.4 mM working solution *p*NPP was added to 10 µl sample and the change in absorbance at 410 nm over 30 min at 25°C was measured in 96 well microplates using a microplate reader (Synergy HT, BioTek).
The optimal temperature and pH of lipase was measured using *p*NPP as substrate. The optimal temperature of the lipase (at pH 8.2) was very broad (30 - 40°C) with a slight peak around 37°C, and the optimal pH (at 25°C) was about 8.5.

In vitro effect of nutrients on enzyme release

The effect of nutrients on the *in vitro* release of lipase from caecal epithelium was tested by the addition of glucose (1 - 4 mg nutrient/ml LGR) and oleic acid (0.05 - 0.1 mM) to the incubation medium (LGR) containing freshly rinsed caeca of one cricket. Lipase activity in the incubation medium without any added nutrients (control) was set to 100 % for comparison to media with nutrients.

Statistical analyses

The SigmaPlot 11.0 program (Systat Software GmbH) was used to evaluate the data. All data were statistically tested for homogeneity of variance (Levene's test) and normal distribution (Shapiro-Wilk test). Paired t-test was used for linked data of the effect of incubation temperature on lipase release. Independent data were evaluated using either ANOVA or Kruskal-Wallis test and individual post hoc analysis. For statistical analysis data of temperature effect (Fig.7) and light-dark cycle (Fig. 5A) on enzyme release were normalized by $\log_{10}$ transformation, data of food uptake presented as crop weight (Fig. 5B) were normalized by square root transformation. The statistical significance is designated in the graphs and text.

Results

Lipase activity was measured in the luminal content, tissue homogenate and secretion of two day old fed *G. bimaculatus*. In females lipase activity was nearly equal in the luminal content and tissue homogenate, but was significantly higher than that secreted over 30 min (Fig. 1A). In male crickets lipase activity showed a similar trend with higher enzyme activity in luminal content (Fig. 1B).

Both, a non nutrient diet (cellulose diet) as well as starvation caused a significantly lower lipase activity in the luminal content and decreased lipase secretion from caecal epithelium. In starved and cellulose fed crickets lipase activity in luminal content was about 90 % less than in fed ones (Fig. 2A), but lipase release was reduced by only about 50 % (Fig. 2B). The *in vitro* effect of nutrients on lipase release was tested by incubation of caecal tissue in low glucose Ringer with the addition of either glucose or oleic acid. There was a dose dependent increase of lipase release in response to oleic acid (0.05 - 0.1mM) (Fig. 3B), but glucose showed no effect (Fig. 3A).

Feeding behaviour in insects is correlated to the quality and availability of food, but also related to ontogenesis. The age-dependent lipase release in *G. bimaculatus* (Fig. 4) showed increasing activity from day 0 to 5 of the last instar larvae. There was no difference in lipase secretion between male and female larvae with a maximum activity of 65-78 nmol pNP/30min at day 5, and the lipase activity decreased from day 5 to 8 (5.9-6.4 nmol pNP/30min). After the imaginal moult lipase release in female crickets increased rapidly from 8 to 100 nmol pNP/30min within five days, whereas lipase activity in males remained low (~30 nmol pNP/30min). From day 6 lipase secretion in males increased reaching maximum at day 7, whereas lipase release in females declined. In general however, there was a higher lipase release in adult females than in males.

Lipase secretion from caecal epithelium (Fig. 5A) and food uptake (crop weight) (Fig. 5B) was followed over a period of one day (24 h) in the last two larval stages of females and in the adults. Crickets were reared at 27°C under a long-day regime with a photophase from 6:00 to 22:00 CEST (see materials and methods). There was a significant effect of stage and time on lipase release. Adult crickets and last instar larvae showed increasing lipase secretion from 4:00 to 24:00 CEST, reaching a maximum (116 - 136 nmol pNP/30min) in the late photophase to early scotophase (22:00-24:00 CEST) (Fig. 5A). In penultimate larvae there was no clear trend of increasing lipase activity over 24 h. The crop weight of crickets was determined at the time of dissection (Fig. 5B). There was a significant effect of time and stage on the crop weight of the crickets with a significant interaction, which indicates differences in feeding behaviour (time of food uptake) within individual developmental stages. The crop weight of last instar larvae and adult crickets was highest at 24:00 CEST, with a higher food uptake to the beginning of darkness (Fig. 5B). In contrast, the crop of penultimate larvae was filled at the end of the scotophase at 4:00 CEST. However, there was no correlation between crop weight and lipase release of any developmental stage (Spearman rank order: $p > 0.05$) (data not shown).

Temperature is one of the most important environmental factors that directly influence the metabolic rate, growth, food consumption and enzyme secretion in insects. At an incubation temperature of either 25°C or 35°C there was no effect on lipase release from caecal tissue of crickets acclimated at 22°C (Fig. 6A) or 32°C (Fig. 6B) from the time of the imaginal moult or those acclimated since the moult to last larval instar. But there were slight but significant differences in females reared since the moult to the penultimate instar. Lipase release increased at 35°C IT when reared at 22°C, but decreased at 35°C when reared at 32°C (Fig. 6A,B). However, lipase release was always higher in crickets reared at 32°C than those reared at 22°C (Fig. 7). Furthermore, there was a significant effect of exposure time to the two acclimation temperatures on lipase release and a strong interaction of rearing temperature and exposure time. Lipase release increased the longer crickets were acclimated at 32°C, but not when acclimated at 22°C (Fig. 6).

Crickets acclimated at 22°C, compared to those acclimated at 32°C from the beginning of the last instar or penultimate instar showed retarded (slower) progress in development the longer they were exposed to the lower temperature (Table 1). Interestingly the total amount of consumed food throughout the last instar did not differ between crickets acclimated at 22°C or 32°C (Fig. 8), but the daily amount of food uptake at 22°C was greatly reduced (Fig. 9). At 22°C food uptake increased over the first three days to 80 - 100 mg food/body weight [g] and stayed the same until day 13 of last instar. Thereafter, feeding gradually decreased until the final moult (Fig. 9A). Food uptake of crickets reared at 32°C on the other hand increased quickly from day 0 to 2 with maximum of 400 mg food/g body weight, and decreased rapidly from day 3 to 6 prior to the final moult (Fig. 9B). After the imaginal moult the daily food uptake within the first three days was three times higher at 32°C (~300 mg food/ g body weight) compared to 22°C (~100 mg/g body weight), and also the total amount of consumed food through adult life was significant higher at 32°C (Fig. 8).

Discussion

Temperature is one of the most important abiotic factors, that influence insect development (time, number of stages, growth rate) and biology (food consumption, metabolic rate, fertility, locomotion, reproduction, digestion), and therefore its life history and phenotypic plasticity (Chown & Terblanche, 2007). Insects are ectothermic and the rate functions of most activities are related to ambient temperature (Hoffmann, 1974; Merkel, 1977, Behrens *et al.*, 1983; Haderspeck & Hoffmann, 1991; Booth & Kiddell, 2007; Lachennicht *et al.*, 2010). Increasing rearing temperature from 22°C to 32°C led to the expected decrease in the duration of the last instar and penultimate instar of *G. bimaculatus*. All reported digestive enzymes have *in vitro* temperature optima, however, these optima often do not correspond to the ambient temperature (Terra *et al.*, 1996). According to Woodring *et al.* (2009) the temperature optimum for lipase in *G. bimaculatus* is about 37°C at an optimal pH 8.0. Incubation temperature of caecal tissue (25°C or 35°C) has no effect on enzyme release in 2-day adults after short-term acclimation (since the beginning of last instar or since adult ecdysis), but there was a slight difference with longer acclimation (since the beginning of the penultimate larval instar), which may indicate a release of different isozymes with higher activity at higher temperatures.

An acclimation of the rate of digestive enzyme secretion has scarcely been investigated in insects (Weidlich *et al.*, 2012). The higher acclimation temperature of 32°C led to a significant increase in lipase release of adult crickets over different exposure times compared to those reared at 22°C. This indicates no acclimation of a rate function in the classical sense, as defined by Prosser (1991). The trypsin secretion in *G. bimaculatus* on the other hand showed a higher rate of secretion after acclimation to 22°C than when acclimated at 32°C (Weidlich *et al.*, 2012), which is the classical positive temperature acclimation pattern. Temperature has a direct stimulatory effect on the amount of lipase released and a higher temperature also appears to stimulate lipase synthesis, in that more lipase is secreted at a higher incubation temperature (35°C compared to 25°C). Moreover, lipase synthesis is strongly influenced by exposure time at different acclimation temperatures. The longer crickets were reared at 32°C, the more lipase was synthesized, stored in endothelial cells and subsequently secreted.

In insects food consumption is correlated to the sex, age, developmental stage, rearing temperature and light-dark cycle, and these factors (via food consumption) also influence the release of digestive enzymes. The secretion of lipase in the caeca of *G. bimaculatus* is similar to that of trypsin, amylase and cellulase (Weidlich *et al.*, 2012, 2013), and is directly related to food uptake (Woodring & Lorenz, 2007). Elevated lipase release in female crickets might be associated with a greater need of lipids for egg production (Espig & Hoffmann, 1985). In both sexes last instar larvae showed a rapid decline of lipase release from day 6 to 8 associated with the preparation of the final moult in that the gut is emptied and much less or no food is consumed (Anand & Lorenz, 2008).

Digestive enzyme release in *G. bimaculatus* depends on both quantity and quality of the diet. Lipase secretion and lipase activity in the luminal contents in starved and non-nutrient (cellulose) fed crickets

was greatly reduced compared to diet-fed crickets. Specific nutrients in the diet stimulate the release of lipase. Oleic acid, for example, when added to the incubation medium of caeca leads to a significant increase of lipase secretion, indicating a prandial release mechanism, similar to that reported for trypsin and amylase (Woodring *et al.*, 2009). Rana & Stanley (1999) already reported a stimulatory effect of the presence of phospholipids on the secretion of phospholipase A_2 in the midgut of *Manduca sexta*. Interestingly, glucose in the incubation medium has no effect on lipase release.

There was a significant effect of photoperiod and developmental stage on lipase secretion in *G. bimaculatus*. Feeding and lipase secretion was highest at the beginning of the scotophase (24:00 CEST) in the last instar and in the adult stage, but for unknown reasons not in the penultimate instar. Last instar larvae and adult crickets are basically nocturnal, in that locomotory activities take place in the scotophase (Nowosielski & Patton, 1963; Nielsen & Dreisig, 1970; Loher, 1972; Tanaka *et al.*, 1999; Lorenz, 2007). The increase of food uptake and lipase secretion was shown to result from an increase of locomotory activity in *Acheta domesticus* (Woodring & Clifford, 1986). A similar effect was also reported for trypsin secretion in *G. bimaculatus* (Weidlich *et al.*, 2012). Both sexes of last instar *G. bimaculatus* show an age-dependent cyclic pattern of activity with maxima during early to mid scotophase and minima during early photophase. After the imaginal moult the crickets show a continuing cyclic of activity until day 6 of adult life (Faßold *et al.*, 2010).

In insects the pars intercerebralis is involved in the circadian regulation of activity levels (Matsui *et al.*, 2009). Studies on *Periplaneta americana* showed increasing locomotion, food consumption, as well as amylase and protease activity in the dark phase (Matsui *et al.*, 2009). Furthermore digestive enzyme release in the insect midgut underlies the influence of neuropeptides (Lehane *et al.*, 1995; Fusé *et al.*, 1999; Harshini *et al.*, 2002a, b; Aguilar *et al.*, 2003; Hill & Orchard, 2005; Sakai *et al.*, 2006; Woodring *et al.*, 2009; Lwalaba *et al.*, 2010).

In conclusion, lipase secretion in *G. bimaculatus* is strongly influenced by endogenous and environmental factors. Higher temperatures associated with longer exposure to different acclimation temperatures lead to an increase of lipase synthesis in caecal tissue and, therefore, to an increased secretion. Lipase secretion is correlated to sex, age, developmental stage, and circadian activity rhythm of the crickets, and all these factors influence feeding behaviour. In general increased food intake results in increased lipase release.

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Figures

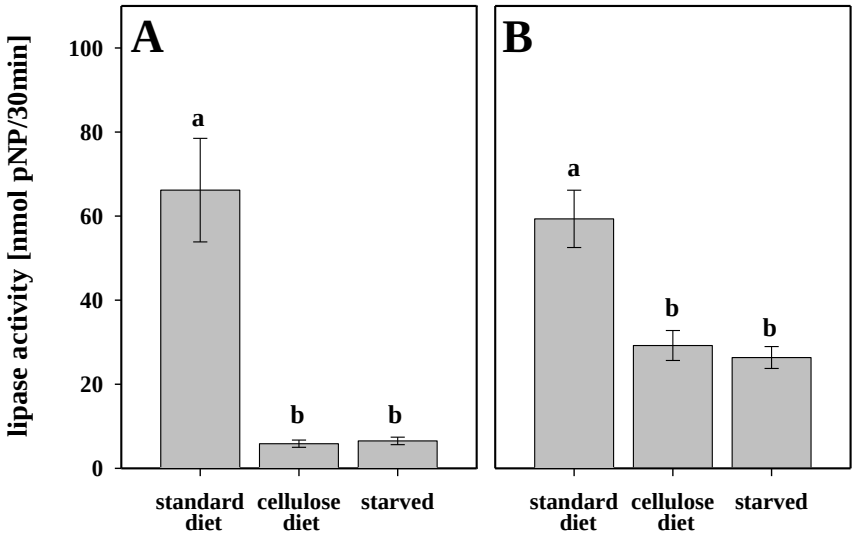

Fig. 1: Lipase activity in luminal content, tissue homogenate and secretion medium of female (A) and male (B) 2-day-old adult *G. bimaculatus* reared at 27°C. Mean ± SEM. n = 10. Statistics: (A) ANOVA ($F_{2,29}$ = 8.885, $p < 0.001$) and post hoc Holm-Sidak method, (B) Kruskal-Wallis test (H= 1.303, df = 2, $p > 0.05$). Different letters indicate significant differences.

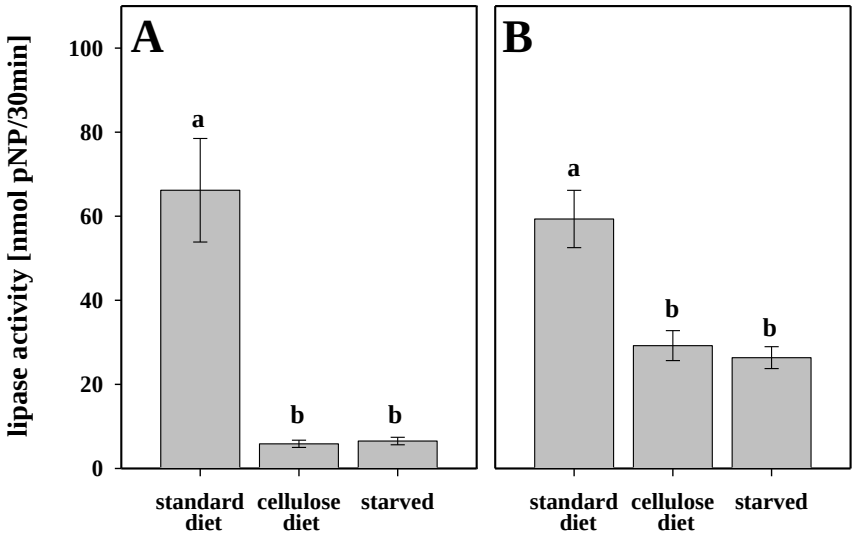

Fig. 2: The effect of starvation and feeding on lipase activity in luminal content (A) and secretion medium (B) in the caeca of 2-day-old adult *G. bimaculatus* females reared at 27°C. Mean ± SEM. n = 18-20. Statistics: Kruskal-Wallis test (A: H = 29.186, df = 2, $p < 0.001$; B: H = 21.745, df = 2, $p < 0.001$) and post hoc Dunn's test. Different letters indicate significant differences.

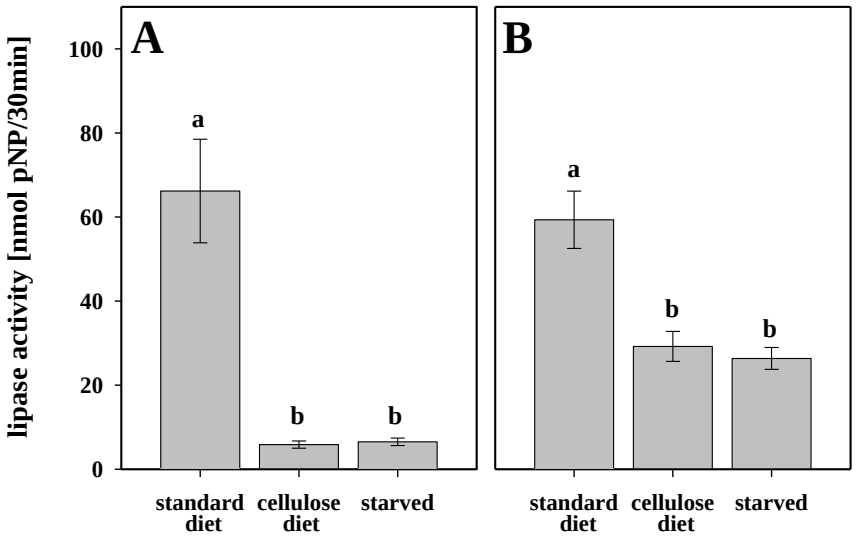

Fig. 3: *In vitro* effect of glucose (A) and oleic acid (B) on lipase release from incubated caeca (37°C, 30 min) of 2-day-old adult *G. bimaculatus* females. Lipase activity of controls (0 mg/ml glucose, 0 mM oleic acid) was set 100 %. Mean ± SEM. n = 10. Statistics: Kruskal-Wallis test (A: H = 3.524, df = 3, p > 0.05; B: H = 19.424, df = 2, p < 0.001) and post hoc Tukey test. Different letters indicate significant differences.

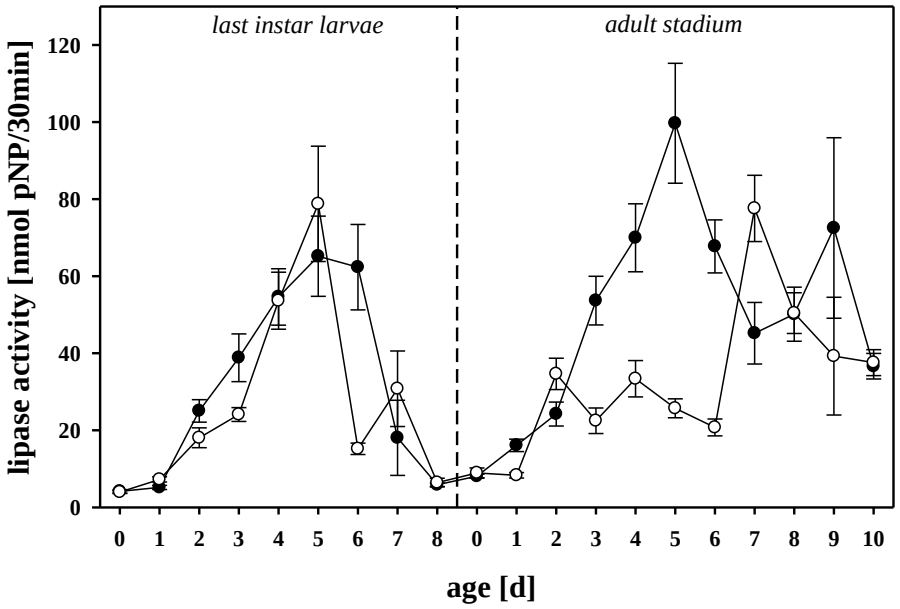

Fig. 4: Age-dependent activity of lipase secretion (30 min incubation at 37°C) of male (○) and female (●) last instar larvae and adults of *G. bimaculatus*. Mean ± SEM. n = 10.

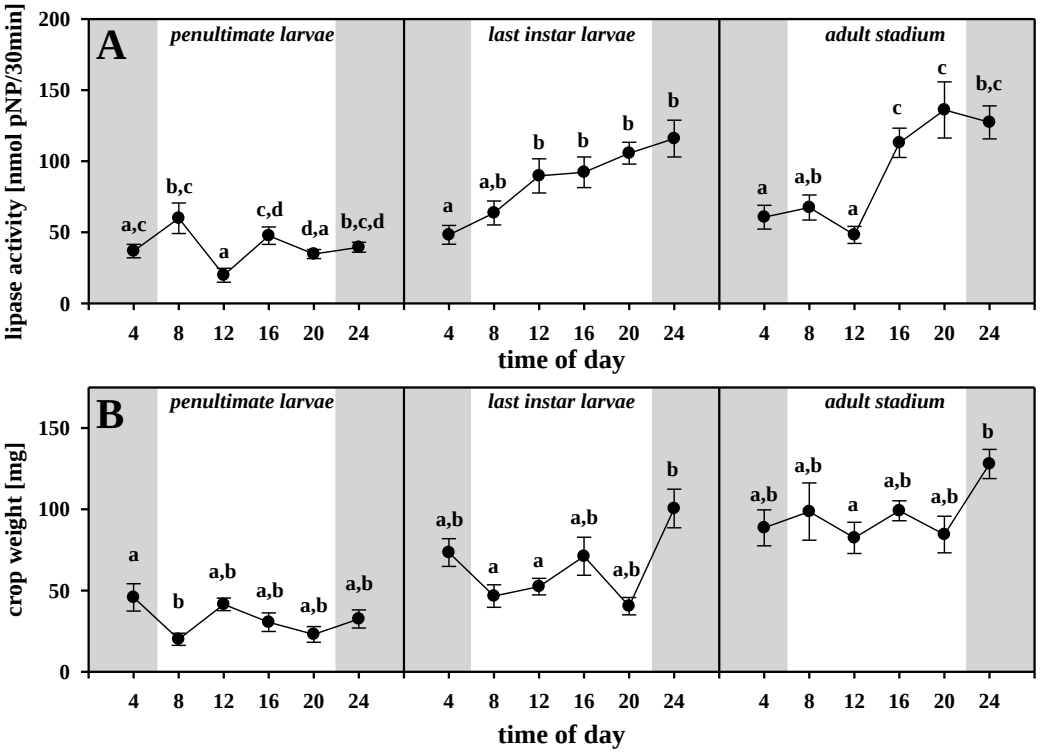

Fig. 5: Lipase release (A) and crop weight (B) of 2-day-old penultimate larvae, last instar larvae and adult females of *G. bimaculatus* over a 24 h period. Scotophase was from 22:00 to 6:00 CEST (grey). Mean ± SEM. n = 9-10.

Statistics: (A) two-way ANOVA: stage ($F_{2,178}$ = 20.09; $p < 0.001$); time ($F_{5,178}$ = 11.64; $p < 0.001$); interaction stage*time ($F_{10,178}$ = 4.76, $p < 0.001$). (B) two-way ANOVA: stage ($F_{2,178}$ = 89.68; $p < 0.001$); time ($F_{5,178}$ = 6.62; $p < 0.001$); interaction stage*time ($F_{10,178}$ = 2.18; $p = 0.022$). Post hoc comparison (Tukey test) for factor time within individual stages. Different letters indicate significant difference.

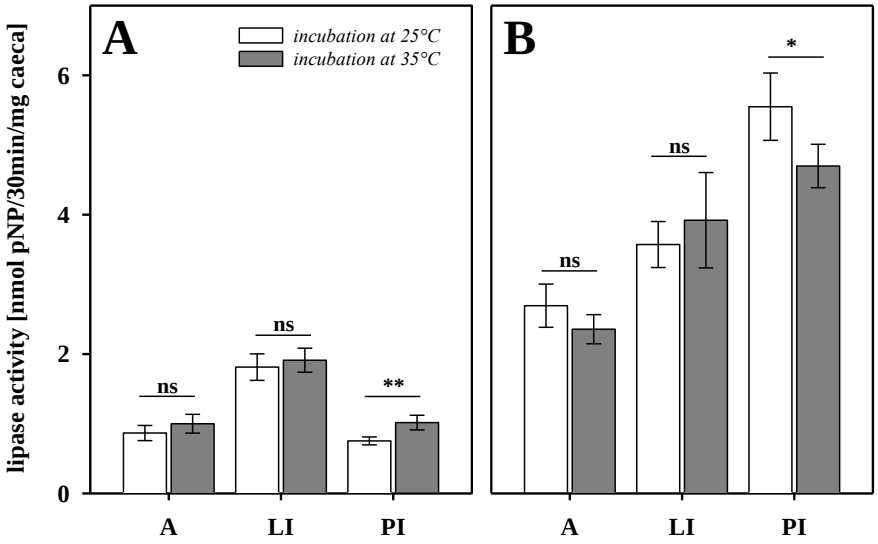

Fig. 6: The effect of incubation temperature on lipase secretion from caecal epithelium of 2-day-old adult female *G. bimaculatus* acclimated at (A) 22°C and (B) 32°C for three different exposure times. A = from day 0 of adult stadium, LI = from day 0 of last instar, PI = from day 0 of penultimate instar. Mean ± SEM. n = 20-40. Statistics: paired t-test. ns = not significant, * = $p < 0.05$, ** = $p < 0.01$.

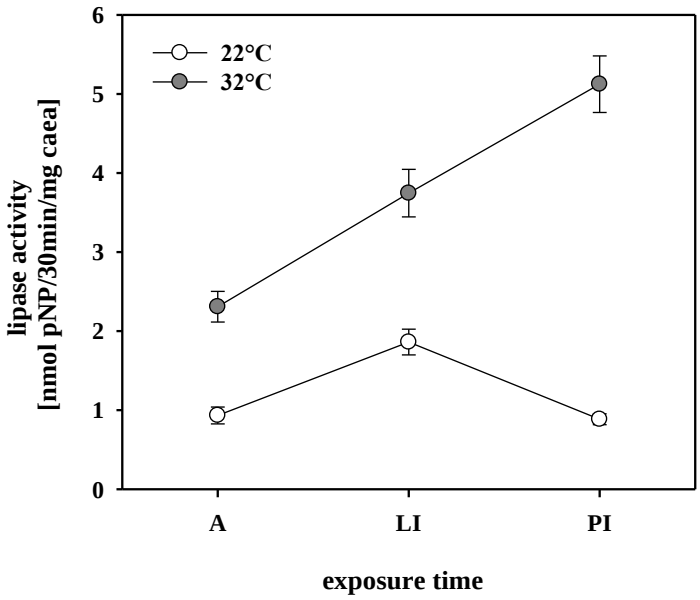

Fig. 7: Lipase secretion from caecal tissue of female *G. bimaculatus* acclimated at 22°C and 32°Cfor three different exposure times (see Fig. 6). Mean ± SEM. n = 19–36. Statistics: two-way ANOVA: temperature ($F_{1,139} = 236.71$, $p < 0.001$); exposure time ($F_{2,139} = 24.5$, $p < 0.001$); interaction temperature*exposure time ($F_{2,139} = 19.54$, $p < 0.001$).

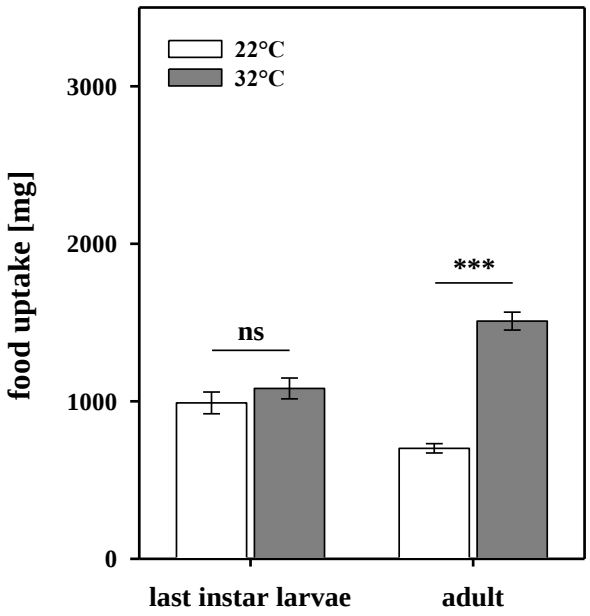

Fig. 8: Average food uptake [mg] of female *G. bimaculatus* in last larval instar and adult stadium. Mean SEM. n = 5-10. Statistics: last instar larvae (t-test: t = -0.863, df = 13, p > 0.05), adult (t-test: t = -9.543, df = 13, p < 0.001),. ns = p > 0.05; *** = p < 0.001.

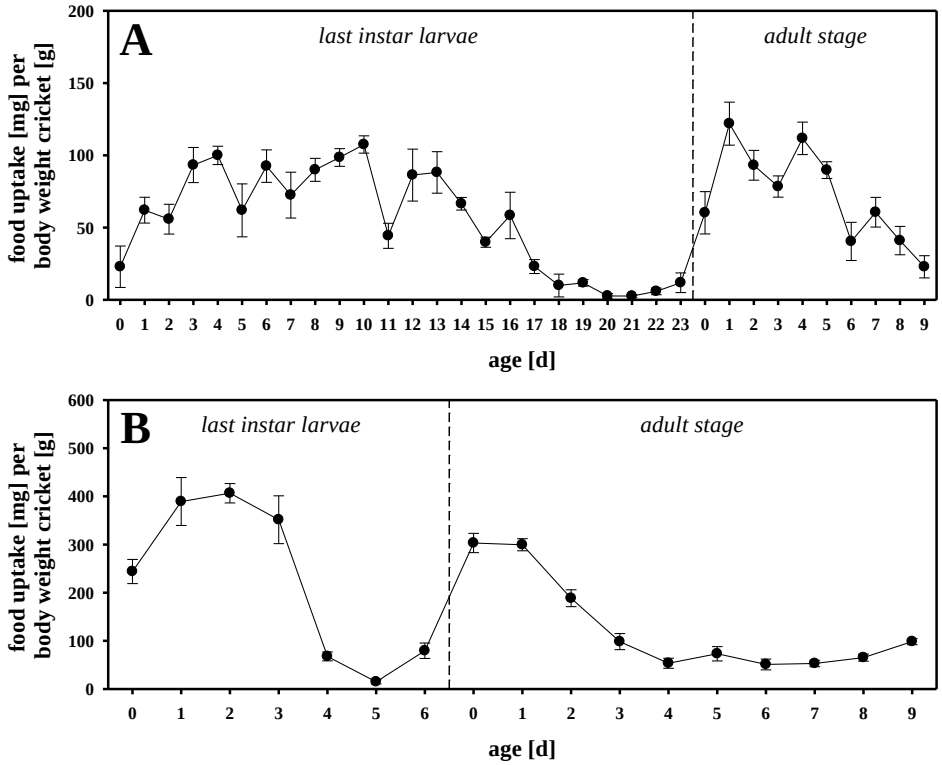

Fig. 9: Age-dependent food uptake of female *G. bimaculatus* through last larval instar and adult stadium acclimated at (A) 22°C and (B) 32°C. Mean ± SEM. n = 5-10.

Tables

Table 1: Duration of the last two larval instars [days] of female *G. bimaculatus* acclimated at 22°C and 32°C and the total exposure time at acclimation temperature until dissection. Mean ± SEM. n = 19-36.

	since penultimate instar		***since last instar***	
	22°C	**32°C**	**22°C**	**32°C**
penultimate instar	15.26 ± 0.20	5.00 ± 0.00	-	-
last instar	18.16 ± 0.23	6.20 ± 0.09	16.37 ± 0.21	6.56 ± 0.13
total exposure time	33.75 ± 1.71	13.20 ± 0.09	17.55 ± 0.84	8.36 ± 0.08

Publication 4

Weidlich S., Hoffmann K.H. and Woodring J.

Activation and autolysis of trypsin in the midgut of the Mediterranean field cricket, *Gryllus bimaculatus*.

will be submitted shortly

Activation and autolysis of trypsin in the midgut of the Mediterranean field cricket, *Gryllus bimaculatus*.

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Abbreviated title: Activation and autolysis of *Gryllus* trypsin

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Abstract

In *G. bimaculatus* the cells of the midgut epithelium synthesize trypsin precursor (TP), which is stored in cytoplasmic vesicles. TP of rinsed trypsin-free caecal tissue was free from vesicles by sonification. The complete self-activation of TP *in vitro* requires about 60 min. The maximum self-activation (measured by hydrolysis of BApNA) was 4-times higher in homogenates from fed crickets compared to starved crickets, indicating a positive influence of feeding on TP synthesis in the epithelial cells. Neither the addition of *Gryllus*-trypsin (from lumen contents) nor bovine trypsin (Sigma), when added to the tissue homogenate, accelerated the maximum activity. The presence of calcium ions in the incubation medium resulted in increased secretion of TP from caecal endothelium, indicating an exocytosis mechanism of release. The trypsin activity of incubated lumen content retained its activity over a period of at least 4 h, whereas a bovine trypsin solution lost 80% of its activity in 30 min. It is suggested that insect trypsin in the digestive tract is protected from autolysis by the presence of peptides, a mechanism long known in mammalian systems,

Key words: digestive enzyme**,** zymogen, trypsin, protease, cricket, autolysis, activation

Introduction

Proteases are enzymes with a wide range of physiological roles, including the digestion of dietary proteins, the removal of damaged tissues, and have highly specialized roles in various activation or inhibitory cascades (Kanost and Clem, 2012). Thus proteases are required in a wide range of vital processes such as digestion, growth, fertilization, immunological reactions, wound healing and cell death (Lazure, 2002).

Serine proteases, especially trypsins, occur in the digestive tract of almost all animals, and there is an extensive literature on the occurrence, distribution, secretion, characteristics, and effects of intrinsic or extrinsic factors regulating the secretion (Applebaum, 1985; Davis et al., 1985; Chapman, 1989; Moffat and Lehane, 1990; Graf et al., 1991; Ramos et al., 1993; Terra and Ferreira, 1994; Terra et al., 1996a,b; Cristofoletti et al., 2001; Woodring et al., 2007, 2009; Weidlich et al., 2012). Inspite of this, there are three rather simple questions concerning insects trypsins that remain inadequately answered. First, what is the secretory mechanism of trypsin in insects? Two, is trypsin secreted as a precursor molecule, that must be activated or is it secreted in an active form? Third, how stable is trypsin once secreted and activated?

Most proteolytic enzymes are indeed synthesized as inactive precursors (deAlbuquerque et al., 2001; Khan and James, 1998; Lazure, 2002), which also enables a spatial and temporal regulation of enzyme activity. Trypsin precursors (TP) of vertebrates are activated by hydrolysis of short polypeptide chains between the amino acids isoleucine and lysine or arginine, and by changing conformation of active substrate binding sites (Ehrmann and Clausen, 2004; Pasternak et al., 1999; Walsh, 1970).Trypsin and TP were early targets of protein sequencing studies in vertebrates, but TP has also been described in some insect species (Davis et al., 1985; Moffatt and Lehane, 1990; Graf et al., 1991; Ramos et al., 1993). Trypsin isoforms of various lengths present in the midgut of *Locusta migratoria* probably indicates the presence of precursor proteins (Lam et al., 2000). In *Musca domestica* membrane-bound and newly synthesized TPs are stored in vesicles of the endothelium, which later on fuse with the plasma membrane releasing their content into the lumen (Lemos and Terra, 1992; Terra and Ferreira, 1994; Jordão et al., 1996).

The regulation of protease activity includes microenvironmental factors (pH, ions), gene regulation, synthesis of specific inhibitors, substrate inhibition, or cascade regulation (Lazure, 2002). Several endogenous serine protease inhibitors have already been identified in various cockroach species (Elpidina et al., 2001a,b; Engelmann and Geraerts, 1980; Vinokurov et al., 2007; Zhuzhikov, 1997), but such endogenous trypsin inhibitors were not found in *G. bimaculatus* (Weidlich et al., 2012). Furthermore, autolysis is an important factor in protease regulation too.

In this study the presence of a putative trypsin precursor in *G. bimaculatus* and its activation was investigated, and the influence of feeding on the synthesis rate was determined. Finally, the autolysis of native *Gryllus*-trypsin was compared to that of bovine trypsin.

Materials and method

Rearing methods

The Mediterranean field cricket, *G. bimaculatus* de Geer (Ensifera, Gryllidae), was raised under a long-day regime (LD 16:8 h photocycle) at 27°C. Newly-emerged females were isolated within one hour after the imaginal moult (before they started to feed) and were designated 0-day old. Crickets received a mixed diet (cricket chow) consisting of ground rabbit, rat and cat food in a ratio of 4:2:1 (w/w), all from Altromin Lage, Germany. The total nutrient value of the chow was 40% carbohydrate, 25% protein, and 6% lipids (Lorenz and Anand, 2004). The optimal growth (maximal weight gain and shortest time) for *G. bimaculatus* fed a diet containing from 10 to 50% casein was achieved with a 30% casein diet (Merkel, 1977). To compare the activation of trypsin precursor in fed and starved *G. bimaculatus*, crickets were placed individually into boxes and provided a cube of an agar-diet (40 g cricket chow + 3.6 g agar + 160 ml water) or no food at all (access to water).

Gut dissection and sample preparation

The crickets were ventrally cut open from the last abdominal segment to the neck. The caeca were removed, cut open and rinsed 3-times with *Gryllus* Ringer (138 mM NaCl, 5 mM KCl, 2 mM $CaCl_2 \cdot 2\ H_2O$, 4 mM Hepes, pH 7.2) (GR). This assured that perhaps more than 95% of the digestive enzymes between the intricate folds of the epithelial tissue were removed. A spontaneous contraction of the external muscles of the caeca led to the formation of an open, cup shaped structure with the lumen side outermost and the hemolymph side inner most(termed a flat-sheet gut preparation; Blakemore et al., 1995). Therefore, both sides of the caecal epithelium were equally exposed to the medium during incubation. The caeca are richly supplied with large trachea (Woodring and Lorenz, 2007) and these remain connected, so that the preparation floats on the surface of the incubation medium.

The role of calcium ions

The rinsed caecal tissue of single crickets was divided in half. One half was incubated in low glucose Ringer (10 mg glucose/100 ml *Gryllus* Ringer)(LGR) either with or without calcium ions at 37°C for 30 min. Afterwards, the tissue was discarded, and the supernatant (incubate) was centrifuged at 2,000 g for 2 min and subsequently assayed for trypsin activity.

Activation of trypsin precursor

Fresh rinsed caeca were transferred to 600 µl 50 mM phosphate buffer (pH 7.2) containing few crystals of N-phenolthioureat (to inhibit phenoloxidase) and were homogenised at the lowest setting for few seconds with ultrasonicator (Branson Sonifier 250). The sample was centrifuged at 11,000g for 10 min, the pellet discarded and the supernatant was frozen at -20°C. The homogenate contained little or no trypsin, but sonication and freezing disrupted all vesicles and thereby released TP. Afterwards, the

supernatant was incubated at 37°C for 4 h and aliquots were tested every 30 min for trypsin activity. The increase of trypsin activity in the sample was therefore a measure of the activation rate of TP. Further activation of TP to trypsin was tested by incubation of tissue homogenate with addition of either *Gryllus*-trypsin (lumen content) or bovine trypsin (Sigma-Aldrich, T 8003) for 2 h at 37°C. The rate of trypsin autolysis was measured by determining the trypsin activity in lumen samples(580 µl 50 mM phosphate buffer, pH 7.2 + 20 µl lumen content) every 30 min during incubation at 37°C for 4 h. The autolysis rate of pure bovine trypsin as well as a mixture of *Gryllus*-trypsin and bovine trypsin was compared.

Enzyme assay

The trypsin activity was measured *in vitro* using α-N-benzoyl-DL-arginine-p-nitroanilide hydrochloride (BApNA) as a substrate, which is quite specific for trypsin. A 10 mM BApNA stock solution dissolved in N,N-dimethylformamide (DMF) was diluted with 50 mM phosphate buffer, pH 7.2 to 1 mM shortly before use and brought to room temperature. Trypsin activity was measured by mixing 50-100 µl samples to 700-750 µl 1 mM BApNA. The change in absorbance at 405 nm in 1 min was determined with a spectrophotometer at 405 nm. Trypsin activity is given as the amount of p-nitroaniline split from BApNA per minute.

Statistics

The SigmaPlot 11.0 program (Systat Software GmbH) was used to evaluate the data. All data were statistically tested for homogeneity of variance (Levene's test) and normal distribution (Shapiro-Wilk test). Data on the effect of calcium ions on enzyme release were evaluated using paired t-test. The individual enzyme activities of samples with calcium free incubation medium were set 100%. The secretion of TP was analysed using paired t-test. Data of TP activation were evaluated using either repeated measurement ANOVA (parametric) or Friedmann-test (non-parametric) including additional post hoc analysis (Tukey-test; Wilcoxon-test + Bonferroni correction). The self-activation of TP was analysed by Kruskal-Wallis test and post hoc Dunn's method due to incomplete dataset. The statistical significance is designated in the graphs.

Results

Secretion of TP

The secretion of TP in *G. bimaculatus* was always higher when incubated in LGR with calcium ions, whereby females showed increases of 100% and males about 40% (Fig. 1).

Activation of TP

Self-activation of TP in fed and starved adult *G. bimaculatus* females was tested *in vitro* by incubating the supernatant of homogenised caecal tissue at 37°C over 4 h, and assaying for trypsin activity every 30 min. Trypsin activity in the tissue homogenate of fed crickets showed significant increase within the first 60 min, reaching plateau phase with maximum activity from 60 to 240 min (Fig. 2B). In starved crickets trypsin activity increased somewhat slower from 0 to 120 min, after which a much lower constant activity level was maintained up to 240 min (Fig. 2A). Thus, after about1 h incubation the total amount of TP synthesized in caecal tissue was activated in samples from fed crickets and after about 2 h in starved crickets. Comparison of trypsin activity between samples of homogenate from fed and starved 2-day-old crickets showed almost equal activity levels at the beginning (0 min) but after 4 h the trypsin activity of fed crickets was three time higher than that of starved crickets. The activation of TP was apparently maximized in 1 h (fed) or 2 h (starved), because addition of more *Gryllus*-trypsin (Fig. 3) or bovine trypsin (Fig 4) did not increase the rate of activation nor the maximum levels.

Autolysis of Gryllus-trypsin and bovine trypsin

The spontaneous decrease in trypsin activity (autolysis) of *Gryllus*-trypsin, when assayed in samples of luminal content over a period of 4 h, did not decrease for 2 h. In fact, the activity actually increased over the first 30 min, but remained constant thereafter for 4 h (Fig. 5).The autolysis of pure bovine trypsin on the other hand was rapid, losing 80% of its activity in only 30 min and declined even further to 2h. Interestingly, when *Gryllus* lumen fluid was added to the bovine trypsin, trypsin activity decreased by only 50% in 30 min and remained stable thereafter.

Discussion

In order to inhibit self-digestion of the gut endothelium, regulation of digestive protease activity is essential for all insects. Early studies suggested the occurrence of inactive forms of the serine protease trypsin in midgut cells of *Aedes aegypti* (Graf et al., 1991), *Drosophila melanogaster* (Davis et al., 1985), *Simulium vittatum* (Ramos et al., 1993) and *Stomoxys calcitrans* (Moffatt and Lehane, 1990). Sequenced trypsin precursors of *Aedes aegypti* seemed to be similar to most insect trypsins, but with differences to vertebrate precursors (Barillas-Mury et al., 1991). This study documented for the first time the presence of a putative trypsin precursor in the midgut endothelium of *G. bimaculatus*.

Digestive enzymes in insects are synthesized in the rough endoplasmatic reticulum, processed in the Golgi complex, packed into secretory vesicles and secreted by the gut endothelium via exocytosis, apocrine or microapocrine processes (Jordão et al., 1999). The release of TP was stimulated up to 100% by the presence of calcium ions in the incubation medium, indicating a calcium-dependent exocytosis secretion mechanism as reported for *Tenebrio molitor* (Cristofoletti et al., 2001).

In order to disrupt epithelial cell structures (vesicles) and release all TP from the endothelial cells, caecal tissues were homogenised and centrifuged to remove all active and putative membrane-bound enzymes. Therefore, the increase of trypsin activity during incubation of homogenate extracts must be caused by self-activation (autocatalysis) of TP. An incubation temperature of 37°C may also have an influence on changing the conformation from inactive to active trypsin, as was indicated for activation of chaperone proteins (Spiess et al., 1999). In the current study both *Gryllus*-trypsin and bovine trypsin stimulated TP activation faster and stronger the higher the amount of active trypsin present, as was also reported for TP activation of *S. calcitrans* with porcine trypsin (Moffatt and Lehane, 1990). Furthermore, the activation of TP was always higher in fed than in starved crickets, strongly indicating a positive influence of feeding on the synthesis rate of TP and, therefore, a higher amount of TP in the secretory granules.

Purified bovine trypsin showed a strong decrease in enzyme activity during 4 h incubation, while trypsin from luminal content did not. Bovine trypsin occurs in an active form (β-trypsin) consisting of a single chain polypeptide (24 kDa). The loss of activity is caused by autolysis of β-trypsin, which is cleaved at Lys^{131}–Ser^{132}, to α-trypsin which is held together by disulfide bridges (according to manufacturer's manual, Sigma). A mixture of *Gryllus*- and bovine trypsin showed a constant decrease activity of 50% after 240 min, suggesting the presence of protective factors in the luminal fluid. *Gryllus*-trypsin alone showed no loss of activity during 4 h incubation and, therefore, no autolysis, strongly indicating components in the lumen content protecting trypsin from proteolytic breakdown. The luminal content consists of a mixture of enzymes, ions and digestion products. Thereby the digestive products of protein degradation are the most important factors protecting vertebrate trypsin from autolysis (Fraser and Powell, 1950; Northrop, 1922). Amino acids on the other hand have no protective action on vertebrate trypsin (Northrop, 1922). Ions also may influence enzyme stability and autolysis (Bier and Nord, 1951;

Gabel and Kasche, 1973; Lazdunski and Delaage, 1965; Sipos and Merkel, 1970; Vajda and Garai, 1981).

In conclusion, *Gryllus*-trypsin is stored as an inactive precursor in the secretory granules of the midgut endothelium, whereby food consumption stimulates the synthesis rate of TP in the caecal tissue cells. TP is released to the midgut lumen by exocytosis and can be activated by self-activation (autocatalysis) or other enzymes. Furthermore, it seems likely that the peptides resulting from protein digestion in the lumen protect *Gryllus*-trypsin from autolysis.

Acknowledgements

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Figures

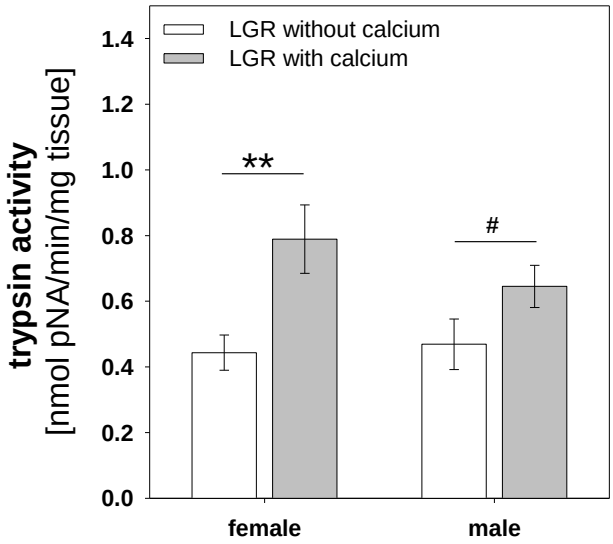

Fig. 1 *In vitro* effect of calcium ions on trypsin secretion from caecal epithelium of 2-day-old *G. bimaculatus* adults. Mean ± SEM. n = 10-15. Statistics: paired t-test: P_{female} = 0.001 (**), P_{male} = 0.051 (#)

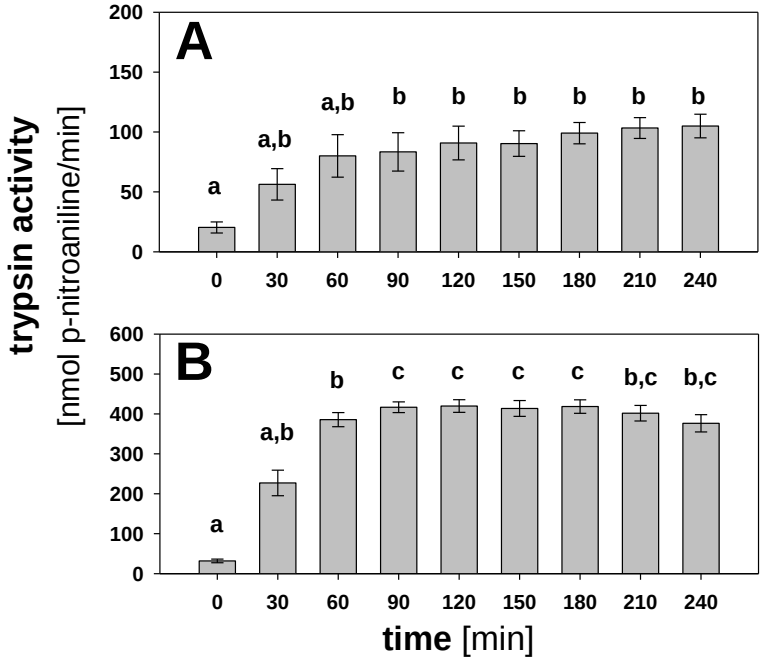

Fig. 2: Self-activation of trypsin precursor from caecal tissue in starved (A) and fed (B) 2-day-old female *G. bimaculatus*. Tissue homogenate was incubated at 37°C for 4 h and aliquots of 50 µl were measured for trypsin activity every 30 min. MW ± SEM. n = 20. Statistics: Kruskal-Wallis test (P < 0.001) and post hoc Dunn's method. Different letters indicate significant differences.

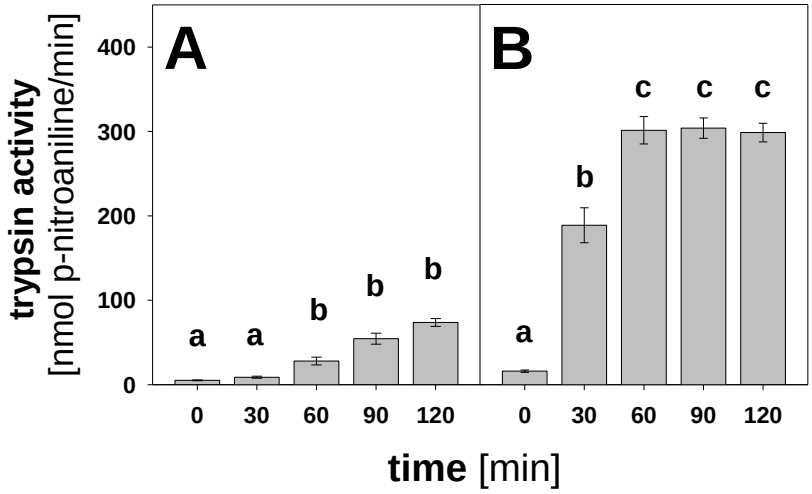

Fig. 3: Activation of trypsin precursor in caecal tissue homogenate of starved (A)and fed (B) 2-day-old adult *G. bimaculatus* females over 2 h at 37°C by addition of *Gryllus*-trypsin from lumen content. Aliquots of 100 µl were taken to measure trypsin activity every 30 min. MW ± SEM. n = 30. Statistics: (A) Repeated measurement ANOVA ($P < 0.001$) and post hoc Tukey-test, (B) Friedman-Test ($P < 0.001$) and post hoc Tukey-test. Different letters indicate significant differences.

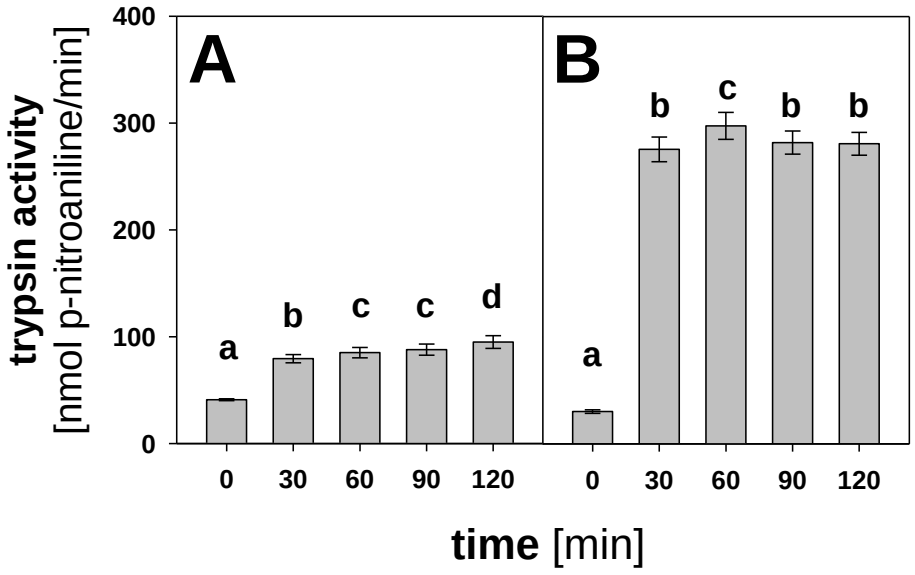

Fig. 4: Activation of trypsin precursor in 2-day-old adult starved (A)and fed (B) female *G. bimaculatus* induced by addition of 20 µl bovine trypsin (1 µg/µl) during incubation at 37°C for 2 h. Aliquots of 100 µl were measured for trypsin activity every 30 min. MW ± SEM. n = 15. Statistics: Friedman-Test ($P < 0.001$) and post hoc Wilcoxon-Test + Bonferroni correction. Different letters indicate significant differences.

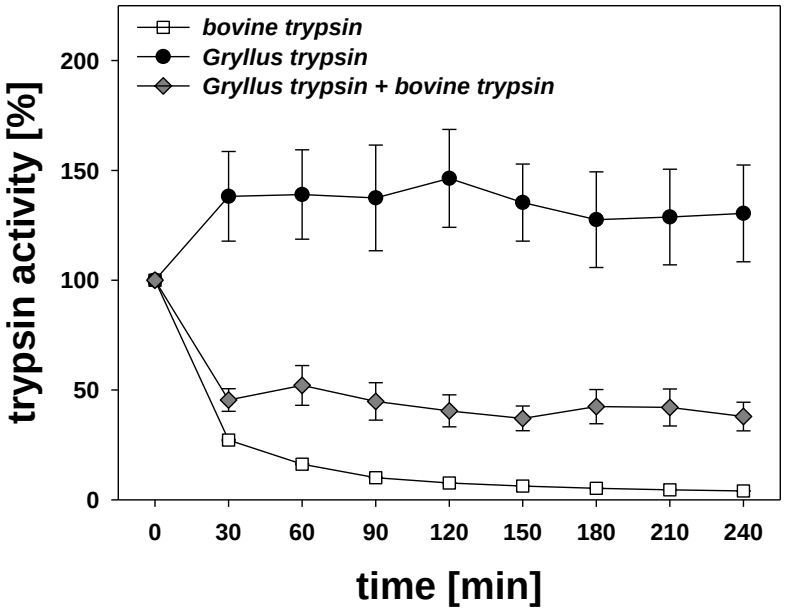

Fig. 5: Activity of *Gryllus*-trypsin, bovine trypsin and a mixture of *Gryllus*- and bovine trypsin during 4 h incubation at 37°C. Aliquots of 50 µl were measured for trypsin activity every 30 min. MW ± SEM. n = 10-20.

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Declaration

Hiermit versichere ich, Sandy Weidlich, die vorliegende Arbeit selbstständig verfasst und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt zu haben.
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I, Sandy Weidlich, declare that this thesis hereby submitted for the Doctor degree at the University of Bayreuth is my own work and has not been previously submitted by me at any another University for any other degree. The work is original except where indicated by special reference in the text and has not been presented to any other University for examination.

Bayreuth, Mai 2013

Sandy Weidlich