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Energy metabolism and enzymatic activity in the *Ips typographus* in relation to diapause.

Ph.D. Thesis

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Annotation

The thesis describes the development and survival of immature *Ips typographus* specimens at low temperatures under laboratory and field conditions. Further, the focus was identifying and characterizing the digestive enzymes present in the gut of adult *I. typographus*, their location in the gut and enzymatic fluctuation over a full calendar year, with a specific focus on digestion of cellulose.

Declaration [in Czech]

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Mgr. et Mgr. Kristýna Štefková

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List of papers and author’s contributions

The thesis is based on the following papers:

I. **Štefková, K.**, Okrouhlík, J., Doležal, P. (2017) Development and survival of the spruce bark beetle, *Ips typographus* (Coleoptera: Curculionidae: Scolytinae) at low temperatures in the laboratory and the field. *Eur. J. Entomol.* 114: 1 – 6. (IF 2015/2016 = 0.86)

II. **Štefková, K.**, Horsley, R., Doležal, P. (manuscript) Cellulolytic activity in the gut of the spruce bark beetle: first evidence of cellulose digestion in *Ips typographus* (Coleoptera: Curculionidae: Scolytinae).

III. **Štefková, K.**, Horsley, R., Doležal, P. (manuscript) Biochemical characterization and compartmentalization of the digestive proteases and carbohydrates of the spruce bark beetle, *Ips typographus* (Coleoptera: Curculionidae: Scolytinae).

Contribution

Kristýna Štefková designed and conducted all the experiments presented in the manuscripts above and was responsible for writing the first draft of these manuscripts.

Co-author agreement

Petr Doležal, the supervisor of Ph.D. thesis and co-author of all presented manuscripts, fully acknowledges the contribution of Kristýna Štefková as the first author and her major contributions according to the statement above.

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RNDr. Petr Doležal, Ph.D.

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CHAPTER I

General Introduction

1. Overview

1.1. Background and context

The European spruce bark beetle, *Ips typographus* (L.), (Coleoptera; Curculionidae; Scolytinae), is one of the most destructive insect pests in European spruce forests and its voracious feeding causes enormous economic and environmental loss each year (Wermelinger, 2004).

In the Czech Republic, *I. typographus* is a significant factor in the damaging of spruce stands. Recent outbreaks in the southern and northeastern parts of the country have attracted significant public and political attention. Uncertainties about its proper management threatening the existence of unmanaged National Parks' core zones, and the status of several protected areas (Furlong, 2006).

Despite its economic importance, our knowledge and understanding of the overwintering capacity and digestive biochemistry of this species has remained limited, nor is anything formally known about seasonal changes in enzymatic activity and how these might relate to key behavioral events throughout the year.

1.2. Aims of the thesis

The primary aims of the thesis were firstly, to observe the maturation and survival of immature *I. typographus* specimens at winter temperatures under laboratory and field conditions; secondly, identify the digestive enzymes present, and their location in the gut of adult *I. typographus;* and thirdly, measure enzymatic activity in the gut of adult *I. typographus* over a full calendar year and consider its correspondence with species typical seasonal behaviors.

1.3. Primary findings and their implications

Findings confirmed that, contrary to previous evidence, all developmental stages (including immature forms) of *I. typographus* can survive low 'winter' temperatures typical of Bohemia in the south of the Czech Republic, and can do so in significant numbers. Further, findings demonstrated, for the first time in this species, the presence of the spectrum of selected gut enzymes from proteinases and glycosidases, focusing on cellulase (necessary for the digestion of cellulose plant material) in

significant quantities in the foregut and midgut of *I. typographus*. Fluctuations in enzymatic activity over the year were demonstrated and approximately corresponded with seasonal behaviors. Whilst more research is needed, detailed understanding of digestion in *I. typographus* may allow feeding-related interventions (such as enzyme inhibition) to be developed that are specific to this pest. Furthermore, knowledge of seasonal effects on digestive activity, temperature effects on development, and over-wintering success may predict *I. typographus* behavior (and *vice versa*), facilitate the specificity and effectiveness of interventions, as well as reduce economic and environmental costs of such interventions.

Taken together, the findings of the present thesis will be important for the control of *I. typographus* and have crucial implications for spruce forest management.

In the introductory (Chapter I), I will present background theory and research on the effect of temperature (and other relevant variables) on over-wintering success followed by a description of morphology and biochemistry of digestion in *I. typographus*. Chapter II is focused on development and survival of *I. typographus* in low temperatures. Chapter III and IV deals with determination and localization of enzymatic activity, its yearly fluctuations, and its potential use in insect pest control programs.

2. Introduction

2.1. Biology of the spruce bark beetle, *I. typographus*

Adult spruce bark beetles are 4.5 – 5.5 mm long, cylindrical, dark brown, and hairy species distributed throughout Europe and northern Asia. Spring swarming depends on temperature and starts in April/May during days with air temperature exceeding 20 °C. In search for a suitable breeding material, emerging individuals attack mainly Norway spruce (*Picea abies*). The number of generations per year depends on climatic conditions (Jonsson et al., 2007; Porter et al., 1991).

Bivoltinism commonly occurs in lowlands (Wermelinger and Seifert, 1999), while populations in higher latitudes (northern Europe) and elevations accomplish only one generation (univoltinism) per year (Jonsson et al., 2007). Up to three generations per year may develop in lower elevations during extremely hot vegetation seasons (Faccoli, 2009).

Mass outbreaks usually occur after large scale forest disturbances caused by wind-storms, heavy snowfalls, severe droughts, or high levels of air pollutants. A surplus of breeding material with little or no resistance to bark beetles, high spring and summer temperatures, and the ability to breed rapidly in damaged/weakened hosts, can result in mass attacks on neighboring healthy trees, substantial economic loss, and enormous environmental damage (Wermelinger, 2004).

2.2. Energy metabolism during hibernation

Seasonality is one of the major abiotic factors that significantly influences the development of insect. Adaptations to the seasonal climatic fluctuations are particularly evident in the species inhabiting the temperate zone (Tauber et al., 1986). The main mechanism used by insect to avoid stressful environmental conditions is ability to induce dormant states, such as diapause. The diapause is a complex developmental and physiological accommodation including a whole range of adaptations, such as changes in behavior and gene expression, a decrease in metabolic rate, and the temporal interruption of development and reproduction (Kostal, 2006). Although the exact termination mechanism of the diapause is not known, a decrease in its intensity and a direct continuation of development may be

brought about by the restoration of favorable outside conditions (Hahn and Denlinger, 2007). However, diapause is often terminated quite a long time before the onset of the conditions suitable for development and reproduction. In such a case, insect stays in the dormant state commonly known as post-diapause quiescence, which lasts until the onset of the suitable conditions (Kostal, 2006).

Most insect don't eat during diapause. Energy reserves gathered before the onset of diapause play a key role in the restoration of normal development and reproduction after its termination (Dekort, 1990). For example, adults of the Colorado potato beetle, *Leptinotarsa decemlineata*, (Coleoptera; Chrysomelidae) do not ingest any food during diapause (Voss et al., 1988). On the other hand, in *I. typographus* when the source of nutrition prevails, it has been proven that the food intake continues during the diapause as well (Dolezal and Sehnal, 2007). Food intake may be also explained by increased temperature in phloem resulting from the direct sunlight (Baier et al., 2007).

The most important sources of energy during the diapause are fats, which are stored in the form of triglycerides in body fat. They can be synthetized through the Krebs' cycle from saccharides and proteins. The main saccharide energy source for insect is glycogen. Other important metabolic reserves are amino acids, which are stored in specialized proteins, called hexamerins (Burmester, 1999; Hahn and Denlinger, 2007).

Energy reserves of insect predictably decrease during the diapause. For example, blow fly *Calliphora vicina* (Diptera; Calliphoridae) (Saunders, 2000) and *Asobara tabida* (Hymenoptera; Braconidae) (Ellers and van Alphen, 2002) decrease in fats linearly with the time spent in diapause. Botterweg (1982) discovered that *I. typographus* loses 40 - 50 % of all its fats during hibernation.

2.3. Effects of temperature

Temperature, a primary driver of insect development, has broad effects on the internal physiology and behavior of insect, including changes in metabolic rate, nutrition, reproduction, flight activity, and survival (Zaslavski, 1988). In mid to high latitudes, temperature is one of the key factor that match the timing of life events with seasonal changes (Stange and Ayres, 2010). Recent increases in the annual temperature averages

caused by climate change (Hansen et al., 2006) have been documented to directly affect population dynamics of numerous insect species (Altermatt et al., 2009; Bale et al., 2002). Overwintered insect emerges earlier (Harrington et al., 2007; Roy and Sparks, 2000), their offspring develop faster, and the occurrence of an additional generation that would normally undergo diapause and overwintering is to be expected (Altermatt, 2010), or has already been recorded (Bale et al., 2002; Jonsson et al., 2007). Although responses may differ in individual species, generally it is expected that climate change will affect insect populations in temperate zones as well (Stange and Ayres, 2010).

Several large-scale outbreaks of bark beetles (Coleoptera; Scolytinae) have been attributed to the warm and dry conditions of the last two decades (Lausch et al., 2013; Williams and Liebhold, 2002). Recent outbreaks in Central Europe have occurred under favorable environmental conditions that followed large scale forest disturbances by wind-storms and severe droughts (Lausch et al., 2013).

Warming climate can shift (depending on altitude) the spring onset towards early April or May and shorten development of *I. typographus* filial generations (Faccoli, 2009). The dominant effect of temperature is overridden by the late-summer shortening of day length that signals seasonal change and causes the induction of imaginal diapause (Dolezal and Sehnal, 2007). However, the completion of development in the last generation is usually not achieved before autumn and a significant part of the population therefore overwinter as larvae or pupae (Faccoli, 2002; Lombardero et al., 2000).

Although several authors have stated that sub-adult stages of *I. typographus* cannot successfully overwinter (Annila, 1969; Coeln et al., 1996; Jonsson et al., 2007); there is a strong evidence that, despite greater susceptibility to mortality than adults, larvae and pupae are nevertheless capable of surviving the winter period (Faccoli, 2002; Zumr, 1982).

2.4. Development of *I. typographus*

Development of insect occurs only when the temperature exceeds the lower developmental threshold (LDT) defined as a “temperature threshold” by which the developmental progress of an insect can be estimated.

LDTs observed in Nordic populations of *I. typographus* were as low as 5°C (Annila, 1969). Central-European studies have described a threshold of 7°C (Vité, 1952) or even higher. Wermelinger and Seifert (1998) presented data based on experiments under constant laboratory conditions, using two methods to calculate the LDT for egg (7.9 and 10.6°C), for larval (8.7 and 8.2°C), and for pupal development (1.6 and 9.9°C, authors suggest the value of 1.6°C is most probably an artefact).

Differences in the LDT may be partially explained by different latitudinal origins of studied populations (Faccoli, 2009) and/or different day-length. Short day conditions hinder the development of larvae and pupae, and may thus affect their acclimatization to low temperature (Dolezal and Sehnal, 2007). The physiological status (active vs. diapause) of specimens may also play a role. Annila (1969) assumed the LDT of 5°C based on a direct observation at low temperature in the field, where fluctuating temperatures may interrupt development. Another possible explanation resides in the ontogenetic history of specimens since LDT may strongly be influenced by cold acclimatization (Terblanche et al., 2011).

Another important indicator of insect development is the so-called effective heat sum, which helps in predicting the population growth in the field. Heat sum is calculated as a sum of positive differences between the diurnal mean temperature and the LDT of given populations. The temperature sum needed for the completion of development ranged between 625–750 degree-days (DD) in northern populations (LDT = 5°C; Harding and Ravn, 1985; Annila, 1969). This value corresponds to the 550-625 DD (LDT = 7°C) recorded in the Central-European population (Netherer and Pennerstorfer, 2001) and the average sum of 572 DD calculated for the beetles in laboratory culture (Wermelinger and Seifert, 1998).

2.5. Functional morphology of the alimentary canal in insect

Insect is extraordinarily diverse in food and feeding habits and hence the digestive systems of different species are similarly heterogeneous in terms of specific gut structure, function, and enzymatic equipment. For example, in most insect, the main area where digestive enzymes are secreted (for the digestion and absorption of nutrients) is the midgut, however in some insect, the main digestive enzymes are found in the fore- or hindgut instead

(Nation, 2008). Despite the diversity of digestion in insect, their alimentary canal is always divided into three separate regions: the foregut (stomodaeum), the midgut (mesenteron), and the hindgut (proctodeum) (Chapman, 1985).

2.5.1.Foregut

The function of the foregut is to take in food, process it mechanically, and prepare it for further chemical digestion. It is composed of the buccal cavity (also mouth), pharynx, oesophagus, crop (also ingluvies), and proventriculus. The buccal cavity is divided into the upper part (cibarium) and the lower part (salivarium), which contains salivary glands. Food digestion starts in the buccal cavity because saliva contains digestive enzymes, mostly amylase (which breaks starch down into glucose) and invertase (which then breaks sucrose down into glucose and fructose) (Nation, 2008).

The pharynx passes the food into oesophagus. Oesophagus is narrow tube, which continues to the proventriculus at the end of foregut. Alternatively, in some insect oesophagus may expand to the crop, which is much enlarged and dilated. Due to its folded architecture, it can extend itself to the necessary size when filled. The crop serves the purpose of storing food and periodically releasing its contents to pass through the proventriculus and into the midgut. In some insect (many Orthoptera and some Coleoptera) (Dow, 1986) substantial digestion may occur in the crop, because digestive enzymes present in gut fluids are regurgitated from the midgut. In the foregut, typically little or no absorption occurs due to an impermeable cuticle.

In species taking in solid food (e.g., cockroaches, termites) the proventriculus is commonly located in the end of foregut and contains sclerotic teeth, ridges, and spines which are intended for pulverizing the food. Alternatively, in other species, the proventriculus may be reduced to a simple valve at the entry to the midgut (Chapman, 1998).

2.5.2.Midgut

The midgut is main site where occurs secretion of digestive enzymes, chemical processing of the food into simpler substances, and subsequent absorption. It is composed of the tubular ventriculus, which contains the

gastric caeca. Additionally, the epithelial cells of the intestinal mucosa contain microvilli. The function of those structures is to greatly enlarge the surface area (for enzyme secretion and for absorption of digested products) of the epithelium and strengthen the digestive and absorptive function (Chapman, 1985).

Also in the midgut is a structure called the peritrophic membrane, which covers processed food preventing direct physical contact between microvilli and food particles. The peritrophic membrane is perforated by small pores, which allow the passage of only small molecules, while the large molecules and food particles stay in the intestine. The peritrophic membrane, thus, creates a semi-permeable barrier which separates different phases of the digestion. It also functions as a barrier to viruses, bacteria, and parasites. It helps prevent rapid excretion of digestive enzymes and compartmentalizes digestion within the midgut (Chapman, 1998).

The middle intestine also includes specialized formations: mycetomes and fermentation chambers, which can contain symbiotic bacteria which help in the processing of food that is difficult to digest (Billingsley and Lehane, 1996).

2.5.3.Hindgut

Finally, the hindgut is divided into ileum (located immediately past the Malpighian tubules), colon, and rectum. In some cases, the ileum grades directly into the rectum (which is terminal part of hindgut) so the colon occurs only in some insect where it is found in the middle region of hindgut. The hindgut plays a major role in reabsorption of water, ions, and dissolved substances from the primary urine washed into the hindgut by the Malpighian tubules (Chapman, 1998).

The hindgut in termites that digest cellulose is more specialized. Their hindgut is noticeably enlarged and can be divided into several morphological parts, which the most important for cellulose breakdown is the colon, where symbionts assisting with the hydrolysis of cellulose are housed (Brune and Friedrich, 2000; Watanabe and Tokuda, 2010)

2.6. Biochemistry of digestion

Digestion is controlled by the secretion of digestive enzymes and is dependent on their localization in the insect gut. In most insect, the midgut is the main location where digestive enzymes are secreted for digestion and absorption of nutrients, however in some insect this is in the foregut or hindgut. Digestion in insect has three phases. Since insect mostly takes food in the form of polymers (e.g., proteins, starch, etc.), the goal of primary digestion is break down polymers into oligomers (which happens inside the peritrophic membrane). During the second phase, oligomers are dispersed to the dimers in ectoperitrophic space, and in the final phase dimers are reduced to monomers (at the surface of midgut cells by integral microvilli enzymes or by enzymes trapped into the glycocalyx) (Terra, 1990; Terra and Ferreira, 2012).

Digestive enzymes[1] belong into the category of hydrolases and they are divided into three classes: peptidase, glycosidase, and lipase. These enzymes are different in their structural dispositions and accordingly they differ in their substrate specificities, sensitivity to specific inhibitors, and by their pH for optimum activity. Below is an overview of the enzyme classes found in insect, major enzymes are described in detail (Terra et al., 1996).

2.6.1. Digestion of proteins

Peptidases (proteolytic enzymes; EC 3.4) are responsible for the digestion of protein, where they completely hydrolyze peptide bonds in proteins. They are divided into two basic categories: endopeptidase (proteinases, EC 3.4.21 – 24) and exopeptidase (EC 3.2.4.11-19).

Endopeptidases break down internal peptide bonds in the polypeptide chain and split the protein into smaller peptide fragments. The most common endopeptidases are trypsin, chymotrypsin, elastase, and pepsin. Exopeptidases attack terminal amino peptide bonds, releasing a single amino acid or dipeptide from the peptide chain. Exopeptidases include enzymes that hydrolyze individual amino acids from the N- or C- terminus

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[1] The nomenclature recommended by Nomenclature committee of the International Union of Biochemistry and Molecular Biology is employed in the present work (Nomenclature Committee, 1992).

of the peptide chain. Aminopeptidases (EC 3.4.11) break down one amino acid remnant from the N- end, carboxypeptidases (EC 3.4.16-18) from the C- end (Rawlings and Barrett, 1994).

There are four subclasses of proteinases: serine, cysteine, aspartic and metallo-proteinases characterized according to their amino acid or metal at the active site of the enzyme (Bode and Huber, 1992).

Serine proteinases: Serine proteinases (EC 3.4.21) have in the active side serine, histidine, and aspartic acid residues and include major digestive enzymes trypsin, chymotrypsin, and elastase. Trypsin (EC 3.4.21.4) cleaves the peptide chain on the carboxyl side of basic L-amino acids such as arginine or lysine. Insect trypsins usually have molecular mass from 30 to 35 kDa and pH optima in the alkaline range (most between 8 - 10). The majority of insect midguts have been shown to contain trypsin-like enzymes (Terra and Ferreira, 1994), with some known exceptions: firstly, in hemipteran species; secondly in taxa belonging to the series Cucujiformia of Coleoptera like Curculionidae, trypsin activity was not found at all (Terra and Ferreira, 2012). Chymotrypsin (EC 3.4.21.1) preferentially cleaves the peptide chain on the carboxyl side of aromatic amino acid. Most insect chymotrypsins have molecular mass from 20 to 30 kDa and pH optima between 8 - 11 with the strong instability at acid pH. The presence of chymotrypsin activity among insect taxa is similar to trypsin activity with some exceptions in some heteropteran bugs (Colebatch et al., 2002). Elastases (3.4.21.36) were found in 1990 by Christeller in the black field cricket *Teleogryllus commodus* and from this time elastase-like enzymes have been characterized in many other insect (Terra and Ferreira, 2012).

Cysteine proteinases: Cysteine proteinases (CPs) (EC 3.4.22) have a catalytic mechanism that requires a cysteine sulfhydryl group. CPs are proteins with a molecular mass between 21 – 30 kDa (Grzonka et al., 2001) and which show their highest hydrolytic activity at pH 5 – 6 (Terra and Ferreira, 2012). The most widespread CPs among animal is cathepsin B (EC 3.4.22.1) (Barrett, 1977) and was first noted in insect midguts (Gooding, 1969). The second well-described cysteine proteinase is cathepsin L (EC 3.4.22.15) (Barrett et al., 1998). CPs are commonly found in the midguts of many insect species (Terra and Ferreira, 1994).

Aspartic proteinases: Aspartic proteinases (EC 3.4.23) have aspartic acid residues in active sites and are optimally active at an acid pH. The most

widespread animal aspartic proteases are pepsin (EC 3.4.23.1) (Fruton, 1976) and cathepsin D (EC 3.4.23.5) (Barrett et al., 2004). The first aspartic proteolytic activity was found in homogenates of whole bodies of house fly *Musca domestica* (Greenberg and Paretsky, 1955) which is subsequently described as cathepsin D occuring in midgut (Lemos and Terra, 1991). Pepsin is rarely, if ever, found in insect (Yadav, 2003). Aspartic proteinases similar to cathepsin D occur in several families of Hemiptera, Heteroptera and in some families of the cucujiform series of Coleoptera. There is an assumption that aspartic proteinases occur together with cysteine proteinases in Hemiptera and Coleoptera (Terra and Ferreira, 1994).

Aminopeptidases: Aminopeptidases cleave amino acids from N-terminus of peptides and belong to the metalloenzymes. Aminopeptidases are divided according to their dependence on metal ions and their substrate specificity, however, in most cases in insect, was found aminopeptidase N (EC 3.4.11.2) which have a broad specificity but preferentially remove a variety of amino acyl β-naphthylamides from peptide chains (Terra and Ferreira, 2012). Insect aminopeptidases have pH optima ranging from 7.2 – 9 and size from 90 to 130 kDa. Aminopeptidases are described for at least six orders of insect (Adang, 2013) and play an important role in digestion in insect because they are usually more active than carboxypeptidases (Terra and Ferreira, 1994).

Carboxypeptidases: Carboxypeptidases cleave C-terminal amino acids from the peptide chain. Insect digestive carboxypeptidases are divided into A (EC 3.4.17.1) and B (EC 3.4.17.2) according to activity in alkaline medium against different substrate. In insect carboxypeptidases A is more widespread, has optimal enzymatic activity in pH range 7.5 – 9, and molecular mass from 20 to 50 kDA. Insect gut carboxypeptidases are widely present in all insect but usually represent a minor component of the protease complement (compared to other hydrolytic enzymes) (Terra and Ferreira, 1994; 2012).

2.6.2. Digestion of lipids and phosphatases in insect

The fats dissociating enzymes (EC 3.1) hydrolyze the ester bond between glycerol and fatty acid. There are three classes of enzyme able to break down ester bonds: (i) carboxyl ester hydrolases (EC 3.1.1) (including lipases, esterases, and phospholipases A and B); (ii) phosphoric monoester

hydrolases (EC 3.1.3) (included phosphatases); and (iii) phosphoric diester hydrolases (EC 3.1.4) (including phospholipases C and D) (Terra et al., 1996).

Lipids containing fatty acids can be divided as storage and membrane lipids (Turunen, 1979). Storage lipids are triacylglycerols and are digested by lipases, while membrane lipids are (i) phospholipids (which are hydrolyzed by phospholipases) and (ii) glycolipids (which are digested by combination of α- and β-galactosidases and triacylglycerol lipases) (Terra and Ferreira, 2012).

The fats dissociating enzymes are much less studied in detail than proteolytic enzymes and glycosidases, often is studied only presence in crude preparations and only a few insect is studied in detail.

2.6.3. Digestion of carbohydrates

Glycosidases (EC 3.2) are classified according to their substrate specificities and are commonly named according to their substrate (Terra and Ferreira, 2012). Glycosidases are very numerous group of hydrolases and below are listed only enzymes which are expected in *I. typographus*, because in insect carbohydrate digesting enzymes depend on diet (Nation, 2008). *I. typographus* feeds on phloem, which contains lignocellulose with cellulose and hemicellulose as the most abundant constituent (Thompson, 1983). Cellulose, hemicellulose, or possibly starch, may be decomposed enzymatically by glycosidases into simpler saccharides, which can serve as a source of energy.

Cellulase: Cellulase provides complete enzymatic degradation of cellulose to glucose and is accomplished by the so-called cellulase complex, i.e. a complex of cellulolytic enzymes with different activities and substrate specificities (Martin, 1983). The cellulolytic complex involves three major classes of glycoside hydrolases: (i) endoglucanases (endo-β-1,4-glucanases, EC 3.2.1.4); (ii) exoglucanases, which include cellobiohydrolase (exo-β-1,4-cellobyohydrolases, EC 3.2.1.91) and exoglucohydrolase (1,4-β-D-glucan glucohydrolase, EC 3.2.1.74); (iii) and β-glucosidases, EC 3.2.1.21 (Clarke, 1997). The effective cellulolytic hydrolysis of native cellulose depends upon the concerted and synergistic action of all three types of glycoside enzymes. Endoglucanase breaks down the inner glycoside bonds along the polyglucan chain, thus, it damages the

crystalline structure of cellulose and, in turn, causes the release of individual polysaccharide chains. Exoglucanases then break away glucose and cellobiose from the end of the polyglucan chain. β-glucosidases complete the final step during cellulose hydrolysis by converting the cellobiose and other short cello-oligosaccharides to glucose from the non-reducing end (Watanabe and Tokuda, 2010).

Xylanase: Xylan is a polysaccharide consisting of xylose residues linked by β-1,4-glycosidic linkages and is most abundant component of noncellulosic polysaccharides – hemicellulose (Moreira and Filho, 2016). The enzymatic hydrolysis of xylan is usually composed by a set of enzymes which include endo-1,4-β-xylanase (EC 3.2.1.8), β-D-xylosidase (EC 3.2.1.37), acetylxylanesterase (EC 3.1.1.6), α-glucuronidase (EC. 3.2.1), and endo- and exo-arabinase (EC 3.2.1.99; EC 3.2.1.55, respectively). All mentioned enzymes act cooperatively to convert xylan into its monomeric units (Polizeli et al., 2005). Hemicellulose is a heteropolymer which contains many different monosacharids, apart from xylose, contains also rhamnose, arabinose, mannose and galactose and for its complete hydrolyze into monomeric sugar is needed complex of many hydrolytic enzymes with diverse modes of action and specificity (Pérez et al., 2002).

Amylase: α-Amylase (α-1,4-glucan-4-glucanohydrolases; EC 3.2.1.1) hydrolyse α-1,4-glucan chains in starch and related carbohydrates to a mixture of maltose, maltotriose, and dextrin (Darvishzadeh, 2014). In most cases, α-amylase has a molecular mass in the range 48 – 60 kDa and the pH optima of the enzyme corresponds to the pH in the midgut specific to the particular insect. Amylase is widely present in insect and starch digestion by amylase already has been found in many insect (Terra and Ferreira, 2012).

α-Glucosidases: α-Glucosidases (EC 3.2.1.20), also named maltases, are responsible for hydrolyze terminal, non-reducing α-1,4-glucosidic linkages from aryl-glucosidases, disaccharides, or oligosaccharides to release a single α-glucose molecule. α-Glucosidases have molecular mass in the range 60 – 80 kDa and pH optima between 5 – 6.5 or pH optima that correspond to the pH value of the insect midgut (Terra and Ferreira, 2012).

2.7. Possible digestion of cellulose in insect

Energy utilization via cellulose digestion is not common in insect. The exceptions are termites which are able to digest cellulose with 74 – 99% efficiency (Martin, 1991). When considering termites, four mechanisms of cellulose digestion have been described. The principle of the first one is that symbiotic bacteria or protozoa decompose cellulose. Termites can also secrete their own cellulase, or, possibly, digest cellulose with help of fungal cellulose ingested with food (Brune, 2014; Martin, 1991).

In the alimentary tract of lower termites cellulolytic flagellate protozoans may be present, as well as diverse populations of bacteria, and endogenous cellulases (Breznak and Brune, 1994; Ohkuma, 2008). Higher termites lack flagellate protozoa and so cellulose digestion is accomplished by a combination of cellulases secreted by gut cells with bacterial enzymes (Brune, 2014). The sites of expression and secretion of endogenous cellulases in lower termites are salivary glands, whilst in the higher termites; cellulases are secreted by the midgut epithelium (Slaytor, 2000). Symbionts (bacteria and/or flagellates) in lower and higher termites dwell in the hindgut (Breznak and Brune, 1994; Ionue et al., 2000; Watanabe and Tokuda, 2010). Until recently there have been described all four mechanisms of digestion of cellulose in many family of low and high termites (Breznak and Brune, 1994; Brune, 2014; Watanabe and Tokuda, 2010).

Whilst generally rare in insect, the ability to break down cellulose (bacterial, fungal, but also by endogenous enzymes) is found in other families of xylophagous beetles. For example, the larvae of European rhinoceros beetle (*Oryctes nasicornis,* Coleoptera: Scarabaeidae) (Bayon, 1980) and the larvae and the adults of linden borer (*Saperda vestita,* Coleoptera: Cerambycidae) (Delalibera et al., 2005) break down cellulose with help of symbiotic bacteria. The digestive enzymes in the middle intestine of the larvae of balsam fir sawyer (*Monochamus marmorator,* Coleoptera: Cerambycidae) are able to break down both hemicellulose and cellulose. Those are enzymes of the fungus *Trichoderma harzianum* and the beetle consumes wood already infected by the fungus (Kukor and Martin, 1986). Furthermore, the endogenous production of enzymes that decompose cellulose and hemicellulose was also found in the larvae and the

adults of yellow spotted longicorn beetle (*Psacothea hilaris*, Coleoptera: Cerambycidae) (Scrivener et al., 1997) and the larvae of *Ergates faber* (Coleoptera: Cerambycidae) (Chararas, 1983). The endogenous production of such enzymes was also found in cockroaches that consume decomposing wood, more exactly in the species of *Geoscapheus dilatatus* and *Panesthia cribrata* (Dictyoptera: Blaberidae) (Zhang et al., 1993).

Insect members of the order Curculionidae (to which bark beetles belong) have likewise demonstrated evidence of cellulase (Martin, 1983; Oppert et al., 2010; Watanabe and Tokuda, 2001), and specifically, these have also been shown in some individual species of bark beetle (Morales-Jimenez et al., 2009, 2012; Valiev et al., 2009; Delalibera et al., 2005; Balogun, 1969).

I. typographus has, similarly as termites, an alimentary canal divided into three parts: the foregut, the midgut, and the hindgut. However, different from termites, which have the hindgut dominant in size (where cellulolytic symbionts are found), the midgut of xylophagous beetles is typically larger (Watanabe and Tokuda, 2010). A relatively small hindgut implies that cellulose digestion in xylophagous beetles is not realized by microbial or protozoan cellulases. However, the only attempt to detect cellulolytic activity in the *I. typographus* has been made by Chararas (1979 in Martin, 1983) with negative results.

2.8. Possible digestion of hemicellulose in insect

Hemicellulose is the second most common substance found in wood after cellulose, and, therefore, it is not surprising that enzymes decomposing xylan were found in xylophagous insect. Xylan degradation was found in lower and higher termites. Among lower termites, enzymes were produced by protozoa in the hindgut (Odelson and Breznak, 1985). Schafer et al. (1996) isolated bacteria able to degrade xylan from the intestinal content of the lower termite, *Mastotermes derwiniensis* that consumed wood. Rouland et al. (1988) isolated two xylanases, the first from the gut of the higher termites *Macrotermes mülleri* and the second from its symbiotic fungus *Termitomyces sp.* Comparison of their kinetic and physical characteristics showed that they were identical. On the other hand, there were two xylanases isolated from the higher termite *Macrotermes bellicosus* (X1T, X2T) and two xylanases from its symbiotic fungus

Termitomyces sp. (X1Mc, X2Mc). Only the xylanases X1T and X1Mc were identical (Matoub and Rouland, 1995).

Previous results show that there are different metabolic strategies for the decomposition of xylan. Although there are not many detailed studies compared to cellulose digestion, it is clear that the decomposition of hemicellulose, concretely xylan, happens with help of symbiotic bacteria, fungus, protozoa, and yeast. But much more detailed work is needed on this class of enzymes that may be important mainly for insect feed on wood (Ni and Tokuda, 2013).

3. Aims and scope of the thesis

Only a few authors focused on the overwintering, food intake, and its subsequent processing of the *I. typographus*. Therefore, not enough information for comparison of newly retrieved data is available. The aim of the presented thesis is to broaden our knowledge about the influence of temperature upon the development and survival of overwintering of *I. typographus* and identify enzymatic system responsible for the digestion of celluloses in the gut tissue.

Present study is divided in two parts:

The main aim of the first part was to investigate the influence of temperature upon the development and overwintering survival of *I. typographus* larvae and pupae. Three constant laboratory temperatures (0, 5 and 10 °C) and two outdoor locations (sunlit and shaded) were chosen to elucidate the overwintering success. Such information may also help predicting the population dynamics and provide a valuable insight into the mechanisms that contribute to relatively high overwintering success of this species. Results of this study were communicated as a paper and have been presented in Chapter II.

In the second part of the present work, we identify and characterize an enzymatic system functioning in digestion in the gut tissue of the bark beetle *I. typographus*. Information about digestion is necessary to understand insect growth and development and also their inhibitors may play an important role in novel biological insecticidal strategies. The focus was on the identifying of the present gut enzyme, including proteinases and glycosidases with more detailed concentrate on digestion of cellulose. The study was primarily focused on determination of pH optima enzymes, determination the activity of digestive enzymes in foregut, midgut, and hindgut, and finally detected activity throughout the year in selected enzymes. The results of this study have been presented in Chapter III and IV.

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CHAPTER II

Development and survival of the spruce bark beetle, Ips typographus (Coleoptera: Curculionidae: Scolytinae) at low temperatures in the laboratory and the field.

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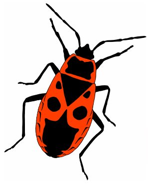

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ORIGINAL ARTICLE

Development and survival of the spruce bark beetle, *Ips typographus* (Coleoptera: Curculionidae: Scolytinae) at low temperatures in the laboratory and the field

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Key words. Coleoptera, Curculionidae, Scolytinae, *Ips typographus*, spruce bark beetle, development, survival, low temperature

Abstract. The European spruce bark beetle (*Ips typographus*) is a highly destructive pest of spruce monocultures. Adult spruce bark beetles are well-adapted to survive over winter however, the ability of sub-adult stages to overwinter has not been clearly established. The increase in average temperature recorded over the last three decades has resulted in an increase in voltinism by one generation, but due to insufficient time the last generation may not complete its development. It is crucial to investigate the survival and development of sub-adult stages at low temperatures in order to predict the effect of increased voltinism on the population dynamics of this species. We measured the development and survival of larvae and pupae (over 12 weeks) in logs kept at winter temperatures outdoors (in shade and exposed to sunlight) and in the laboratory (at 0 and 5°C), with 10°C as a control, at which normal development was expected. Overall, findings revealed that development continued at low temperatures, although it was slower than at high temperatures. Importantly, after 12 weeks significant numbers of spruce bark beetles were present, including newly emerged adults. We demonstrate, for the first time, that sub-adult spruce bark beetles can mature over winter and the percentage survival was significant, indicating that some of the beetles that did not complete their development before the onset of winter can complete their development during winter and potentially adversely affect forests and pose problems for their management.

INTRODUCTION

In Europe, the spruce bark beetle, *Ips typographus* (L.) is the most destructive pest of spruce monocultures. Recent outbreaks in Central Europe have occurred following significant disturbances to forests caused by wind-storms and severe droughts (Zenáhlíková et al., 2011; Lausch et al., 2013 b). An abundance of trees with little or no resistance to spruce bark beetle attack, warmer springs and summers resulting from climate change (Hansen et al., 2006), together with the ability of the bark beetle to breed rapidly in the bark of recently damaged/weakened trees, have resulted in mass attacks on neighbouring healthy trees and enormous economic losses and environmental damage (Wermelinger, 2004; Liška et al., 2016).

The speed of insect development is dependent on ambient temperature and the sum of effective temperatures, which can be used to predict population growth in the field. The sum of effective temperatures (SET) is the sum of positive differences between the diurnal mean temperature and the zero-developmental point of a given species. Zero development point is defined as the "the lower developmental threshold" (LDT) below which the development of an insect ceases. The SET for the complete development of the spruce bark beetle (*I. typographus*) ranges between 550–750 degree-days (Annila, 1969; Harding & Ravn, 1985; Wermelinger & Seifert, 1998; Netherer & Pennerstorfer, 2001). Differences in SET may be explained by differences in the LDT. Temperature, therefore is a primary driver of development; it has broad effects on the internal physiology and behaviour of insects, including changes in metabolic rate, nutritional uptake, reproduction, flight activity and survival (Zaslavski, 1988). In mid to high latitudes, temperature is one of the key cues that synchronizes life cycle events with seasonal changes (Stange & Ayres, 2010). Large scale outbreaks of bark beetles (Coleoptera: Scolytinae) are attributed to the increasingly warm and dry conditions recorded over the last two decades (Williams & Liebhold, 2002; Lausch et al., 2013 a). A possible factor contributing to spruce bark beetle outbreaks is that with increase in temperature, overwintered insects emerge earlier in spring, filial generations develop faster and an additional generation is likely, which normally would undergo

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diapause development and overwinter (Faccoli, 2009). However, not all of the last generation complete their development before autumn and overwinter as larvae or pupae (Lombardero et al., 2000; Faccoli, 2002). Although several authors state that sub-adult stages of *Ips typographus* cannot successfully overwinter (Annila, 1969; Coeln et al., 1996; Jönsson et al., 2007); there is strong evidence that, despite the fact that larvae and pupae are less likely to survive than adults, nevertheless they are able to survive, especially when winters are mild (Zumr, 1982; Wermelinger & Seifert, 1999; Faccoli, 2002; Dworschak et al., 2014). The aim of present study was therefore to investigate, over a period of 12 weeks, the development in terms of an increase in the numbers of live sub-adult beetles and concomitant decrease in numbers of larvae and pupae of *Ips typographus* at low temperatures (in a laboratory and at outdoor locations) in order to determine the overwintering ability of this species. Winter temperatures were varied by placing the logs outdoors in the shade and exposed to sunlight. Since it was not possible to predict accurately what outdoor temperatures would be during the study, three additional laboratory control conditions were used: 0 and 5°C, which should mimic outdoor winter conditions, and 10°C at which normal development and survival is known to occur.

MATERIAL AND METHODS

Experimental design

A repeated measures experimental design with one factor, weeks, which had four levels (0, 2, 6, and 12) was used to determine development over time in each of the temperature conditions (shade, sun, 0, 5 and 10°C). The total number of individuals (living or dead), their developmental stage (larvae, pupae, adults) and other indicators of development such as the numbers of freshly emerged adults (pale coloured beetles) was recorded per dm^2 of phloem. To determine survival, the number of live individuals (larvae, pupae, adults) per dm^2 of phloem was recorded after 12 weeks. All the infested spruce logs were collected from three trees (approximately 80 years old) in November 2013 in the Šumava National Park, Czech Republic, at 1000 m a.s.l. Each log was approximately 50 cm long and 25 cm in diameter with similar quality bark and was similarly infested with larvae, pupae and adults of the spruce bark beetle.

Sample preparation

Logs were transported to České Budějovice and placed in the garden of the institute. Within 24 h of the transfer to České Budějovice, 10 cm wide stripes of the bark were removed from around the circumference of each log and the number of individuals and their developmental stage (larvae, pupae, adults) was recorded and recalculated in terms of area of phloem. The logs were placed into cooled incubators (Sanyo MIR 153 and 253: Sanyo Electric, Osaka, Japan) in the laboratory or outdoors (in sunlit or shaded locations). Logs were sampled and debarked and after 2, 6 and 12 weeks.

Outdoor conditions

In the garden of the Institute of Entomology in České Budějovice, seven logs were stored outdoors in an area exposed to direct sunlight and seven in the shade. During the experiment, the air temperature was recorded at hourly intervals at both outdoor locations using Cometter data loggers (Comet Systems, Rožnov pod Radhoštěm, Czech Republic). Thermal sums were expressed as degree-days (DD) above the lower developmental threshold (LDT), which was set at 8.3°C for all life stages (according to Wermelinger & Seifert, 1998) and/or to 5°C (according to Annila, 1969).

Laboratory conditions

A total of eighteen logs were placed in cooled incubators at constant temperatures of either 0, 5 or 10°C. The incubators were not supplied with artificial light in order to mimic the conditions of snow cover. The logs were checked twice a week and moistened with tap water to prevent the phloem drying out.

Data analysis

Statistical analyses were conducted using STATISTICA v. 12 software (StatSoft Inc., OK, USA, 2013). All data were tested for normality and homogeneity of variances using the Kolmogorov-Smirnov and Levene's test. To test the development over time, a series of one-way ANOVAS (Analysis of Variance) were conducted with weeks (0, 2, 6, 12) as a repeated measures factor and beetle density (measured as the number of individuals per dm^2 of phloem) as the dependent variable. To test survival, the number of living individuals at 12 weeks was compared against a test statistic of zero, using a single sample t-test. In all cases, the alpha criterion for rejection of the null hypothesis was set at $p = 0.05$, and all statistical tests were two-tailed, unless otherwise stated.

RESULTS

Sub-imaginal development of overwintering spruce bark beetles

In mid-November (when logs were collected), the logs contained all the developmental stages of *I. typographus*. The overwintering population in all the logs consisted predominantly of larvae (3.3 ± 1.5 larvae/dm^2) and pupae (4.2 ± 1.9 pupae/dm^2). Adults were less abundant (1.7 ± 0.6 adults/dm^2). Two weeks later, there was no significant differences in beetle density regardless of the temperature (cooled incubators set to 10°C, 5°C and 0°C: Fig. 1) or outdoor storage conditions (sunlit and shaded: Fig. 2). The proportions of the different developmental stages did not change, compared to that at the beginning of the experiment.

Development in outdoor conditions

The number of adults significantly increased in the logs exposed to sunlight outdoors ($F_{3, 18} = 6.307$, $p = 0.004$). There was an increase from 2 ± 0.1 adults/dm^2 (week 0) to 3.7 ± 0.1 adults/dm^2 (week 12) (Fig. 2). The differences recorded in the numbers of spruce bark beetles in the logs in the shade were not significant ($F_{3, 18} = 0.23$, $p = 0.874$). The only indication of possible ongoing development was the presence of newly emerged beetles (0.5 ± 0.07 adults/dm^2 after 12 weeks). Daily air outdoor temperature averages during the experiment exceeded the LDT of 8.3°C only once (Fig. 3).

Development in the laboratory

The fastest development of beetles was recorded at a constant temperature of 10°C, with only adult beetles present after 6 weeks (Fig. 1) and the average number of adults significantly increased from 1 ± 0.06/dm^2 at the beginning (0 weeks) to 4.4 ± 0.1/dm^2 at 6 weeks ($F_{2, 10}$ =

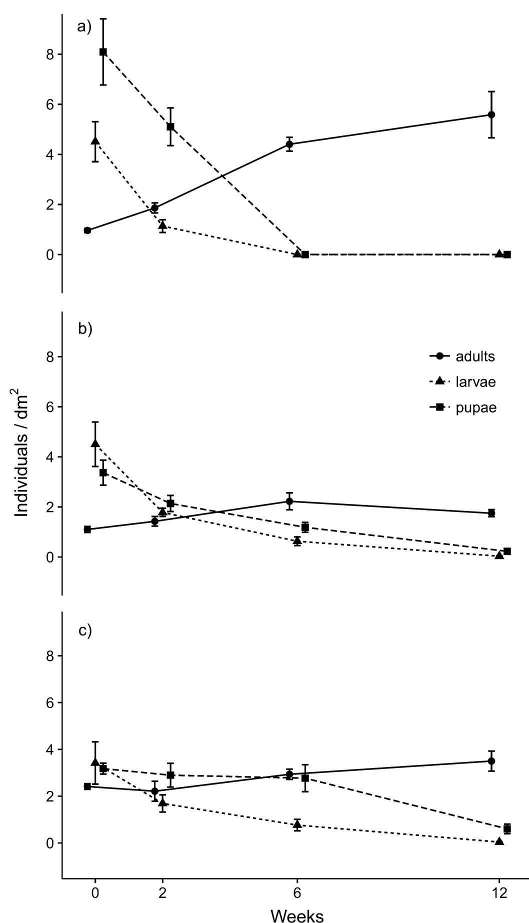

Fig. 1. The numbers of larvae, pupae and adults of the spruce bark beetle, *Ips typographus*, per dm² recorded in logs kept at 10 (graph a), 5 (graph b) and 0°C (graph c) after 0, 2 ,6 and 12 weeks. Data-points are the numbers and standard deviations recorded. Both living and dead individuals were included.

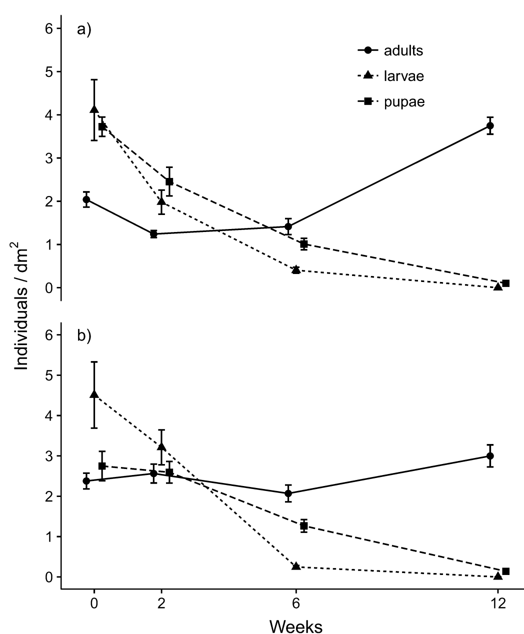

Fig. 2. The numbers of larvae, pupae and adults of the spruce bark beetle, *Ips typographus*, per dm² recorded in logs kept for 0–12 weeks outdoors exposed to sunlight (graph a) and in shade (graph b) in the garden of the Institute of Entomology, České Budějovice. Data-points are the numbers and standard deviations recorded. Both living and dead individuals were included.

8.117, $p = 0.008$). A marginally significant increase to 5.6 ± 0.6 adults/dm² was recorded after 12 weeks ($F_{3, 15} = 2.884$, $p = 0.071$) in 10°C. At that time, newly emerged beetles made up 78% of all the adults in the logs.

No significant changes in the density of adults were recorded at 0°C ($F_{3, 12} = 0.539$, $p = 0.665$) or 5°C after 12 weeks ($F_{3, 15} = 0.346$, $p = 0.297$). However, newly emerged beetles were recorded at both temperatures after both 6 and 12 weeks (Fig. 1). At 0°C, the proportion of yellow col-

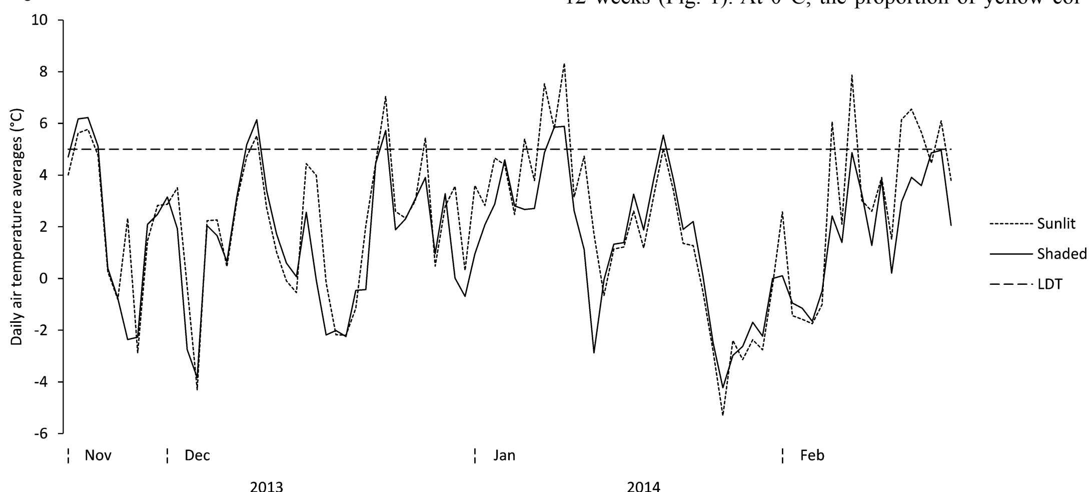

Fig. 3. Daily average air temperatures (°C) recorded during the winter 2013/2014 (November to February) at the two outdoor locations: (A) exposed to sunlight, and (B) in shade. Data loggers were placed at a height of 1.5 m close to where the logs were kept.

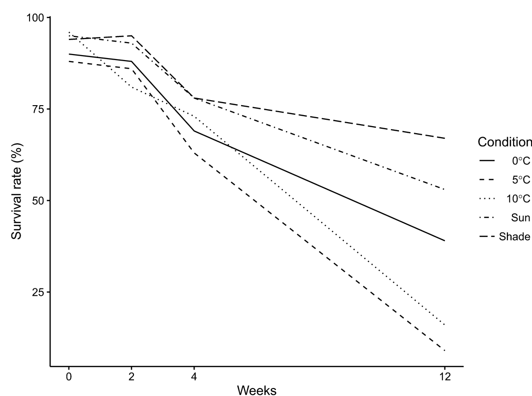

Fig. 4. The percentage of individuals alive after 0, 2, 4, 12 weeks when kept at either 0°C, 5°C, 10°C or outside exposed to sunlight and in shade.

oured adults (callow adults) increased from 0.9 ± 0.2/dm^2 at the beginning to 2.5 ± 0.3/dm^2 at the end of the experiment.

Temperature-dependent survival

Development in all the treatments was characterized by a dwindling number of larvae and a stable number of pupae (Figs 1–2). No dead larvae or their remnants (head capsules etc.) were recorded. The highest percentages of survival of all individuals (larvae, pupae, adults) was recorded in the logs kept outdoors exposed to sunlight (84%) and in shade (79%). The percentage survival of adults after 12 weeks was 67% in logs exposed to sunlight. In contrast, the lowest percentage survival of adults of only 9% was recorded at 10°C after 12 weeks (Fig. 4).

After 12 weeks, the number of live individuals (larvae, pupae and adults, both callow and mature) was recorded in each treatment (0°C; 5°C; 10°C; sun; shade) (Table 1). Table 1 shows that beetles survived in all treatments; the highest numbers of individuals were recorded in logs kept outside and exposed to sunlight, followed by those in the shade and kept at 0°C. Smaller numbers were recorded at 5 and 10°C. It is clear that at 0°C in the laboratory, and at winter outdoor temperatures, spruce bark beetles are able to survive; to test this statistically, single sample t-tests were conducted for each treatment to determine whether significant numbers of individuals survived (total number surviving). These showed that there were significant numbers of survivors in the logs in the sun ($t_{(6)}$ = 4.37, p = 0.005), in the shade ($t_{(5)}$ = 3.27, p = 0.022), at 5°C ($t_{(5)}$ = 5.00, p = 0.004) and at 10°C ($t_{(6)}$ = 2.68, p = 0.04), despite problems with the phloem. At 0°C, significant numbers also survived ($t_{(4)}$ = 2.45, p = 0.03, one tailed). Table 1 indicates that similar numbers survived in the shade as at 0°C and in the sun; this was shown to be the case since (unplanned) independent t-tests showed no significant differences between shade vs. sun, or between shade vs. 0°C (maximum $t_{(11)}$ = 0.813, p = 0.43).

Table 1. Mean number of larvae, pupae, mature adults, callow adults and total number of individuals present after 12 weeks when kept at either 0°C; 5°C or 10°C and outside exposed to sun or in shade.

	Larvae	Pupae	Dark adults	Light adults	Total
0°C	0.2 (0.45)	2.6 (5.81)	1.6 (2.07)	3.6 (3.65)	8 (7.31)
5°C	0.17 (0.41)	0.17 (0.41)	0.83 (0.75)	0.50 (0.84)	1.67 (0.82)
10°C	0.00 (0.00)	0.00 (0.00)	2.14 (2.12)	0.00 (0.00)	2.14 (2.12)
Sunlit spot	0.00 (0.00)	0.43 (0.79)	8.14 (4.88)	2.29 (4.35)	10.86 (6.57)
Shaded spot	0.00 (0.00)	0.33 (0.82)	6.17 (4.4)	1.5 (2.81)	8 (6)

Table 2. The sum of effective temperatures (SET) in °C calculated for those individuals kept at 10°C and outdoors exposed to sun and in shade. Both the lower developmental threshold (LDT) of 8.3°C (Wermelinger & Seifert, 1998) and 5°C (Annila, 1969) were used.

	Effective heat sums (°C)	
	LTD = 8.3°C	LTD = 5°C
10°C	142.8	450
Sunlit spot	0.3	19.88
Shaded spot	0	6.86

Sum of degree-days

Inferential analyses of sums of degree days are not presented. Having recalculated the recorded temperatures to daily averages, the averages exceeded the lower developmental threshold (8.3°C) only in one case (10th January 2014), in logs exposed to sunlight. At the constant temperature of 10°C, the sum of degree days after 12 weeks reached 142.8 DD. At that time, all the spruce bark beetles had completed their development and were adults. The substitution of the LDT 8.3°C by LDT 5°C slightly increased the sums of degree days to 19.88 DD for the logs in sunlight, 6.86 DD for those in the shade and 450 DD for those kept at 10°C (Table 2).

DISCUSSION

As previously documented (e.g., Zaslavski, 1988; Hui, 1994; Wermelinger & Seifert, 1998), our experiments confirm that at constant temperatures the rate of development is dependent on temperature. As expected, at 10°C, all larvae and pupae became adults within 6 weeks. After 12 weeks, the stage of development was not significantly different from that at 6 weeks. However, at warmer temperatures in the laboratory the mortality of newly emerged adults was high due to the drying out of the phloem (despite frequent moistening), which may have disrupted feeding. Lower temperatures of 5°C and 0°C suppressed development and immature stages were still present after 12 weeks. However, at the end of the experiment newly emerged adults were recorded at both 0°C and 5°C, which indicates that development occurred at these temperatures in the laboratory. At 0°C, the percentage of freshly emerged adults increased from 38% at the beginning (week 0) to 71% of all adults/dm^2 after 12 weeks. Under outdoor conditions, there was significant development of the beetles in the logs exposed to sunlight; almost exclusively only adult spruce bark beetles were recorded under the bark after 12 weeks. Contrary to previous suggestion (Wermelinger & Seifert, 1999), the density of living adults doubled to 3.7 per dm^2 in the logs exposed to sunlight. The young beetles developed predominantly from pupae because the decrease in the number of larvae was not correlated with the number of pupae. Obvi-

ously, not all the dead larvae were recorded, which can be attributed to both unsuccessful overwintering and intraspecific competition (Lawson, 1993). Development was less apparent in the logs in shade, here, the density of adults increased, but not significantly; even so, there were freshly emerged beetles present. Air temperature did not differ between the logs exposed to sunlight and in shade, therefore such differences in development in the two outdoor treatments could be explained by solar irradiation increasing phloem temperatures resulting in faster development regardless of air temperatures (Harding & Ravn, 1985; Baier et al., 2007; Berec et al., 2013). To summarize, there was some evidence of development in all treatments; this was most clear at 10°C in the laboratory and in logs exposed to sunlight outdoors. At 0, and 5°C, and in logs in shade, development was slower, but still occurred as newly emerged beetles were recorded.

Our findings suggest that spruce bark beetles are able to survive winter temperatures as live beetles were recorded at 0 degrees in the laboratory and more importantly in logs kept outdoors exposed to sunlight and in shade. Moreover, statistically significant numbers of beetles were recorded at 0 degrees, in the logs exposed to sunlight and in shade. Facolli (2002) records that the 49% mortality was mainly due to the death of young adults at the beginning of a harsh winter season. Dworschak (2014) records 39% mortality for populations that consisted of immature stages. The winter mortality recorded in the study of Dworschak (2014) is based only on the number of adults recorded at the end of experiment, whereas our study included all developmental stages. When we re-calculated our results using the methods of Dworschak, our results for logs kept outdoors are similar. Our study provides clear evidence that immature spruce bark beetles can survive winter temperatures, and at numbers that are significantly different from zero.

Our results indicate that the immature stages of polyvoltine Central European populations of the spruce bark beetle can overwinter. The results also indicate that the laboratory conditions were not optimal for this beetle, because relatively few survived at 5 and 10 degrees (most likely due to the drying out of the phloem), which limited the between treatment comparisons.

It is also evident that at temperatures above zero this beetle can continue developing slowly, which is of crucial importance since it indicates that, contrary to previous thinking (Annila, 1969; Bakke, 1983; Coeln et al., 1996; Jönsson et al., 2007) immature stages may be able to survive harsh winters and therefore contribute considerably to subsequent spring infestations. The SETs required for development to the adult stage recorded in this study are lower than those recorded by previous authors (Annila, 1969; Harding & Ravn, 1985; Wermelinger & Seifert, 1998; Netherer & Pennerstorfer, 2001). This discrepancy may be due to differences in the origins of the populations studied. Temperature requirements of pre-diapausing generations may differ from that of normally developing generations, which indicates the need for further studies. Moreover, winters in Scandinavia are usually much longer and colder than in Central Europe (Annila, 1969; Jönsson et al., 2007). In general, the winter 2013/2014 was dry and mild. Logs were covered with snow on only two or three occasions and melted usually within a week. The average monthly temperatures were about 2 to 5°C above the long term averages (1961–1990).

The results of the present study indicate that immature stages of the spruce bark beetle, *Ips typographus*, can continue developing and successfully overwinter and may significantly contribute to spring swarming in Central Europe. Current legislation requires that infested trees are felled at the end of the vegetative season (October or November), when they are mainly infested with sub-adult spruce bark beetles, and in mountainous regions and at locations that are difficult to access (and) should be removed, at latest, the following spring. Such trees are not currently considered a threat to plantations due to the previously supposed high winter mortality of larvae and pupae. The results of this study indicate that the role of newly emerged beetles cannot be neglected and that current forestry strategy should be modified to enforce the removal of all infested timber from the forest as soon as is possible.

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CHAPTER III

Cellulolytic activity in the gut of the spruce bark beetle: first evidence of cellulose digestion in Ips typographus (Coleoptera: Curculionidae: Scolytinae).

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(Manuscript)

Cellulolytic activity in the gut of the spruce bark beetle: first evidence of cellulose digestion in *Ips typographus* (Coleoptera: Curculionidae: Scolytinae).

Abstract

Our study determined the level of cellulolytic activity in the spruce bark beetle, *Ips typographus*. Cellulase activity was examined using fluorescent substrates: (i) in different pH values; (ii) in foregut, midgut, and hindgut; (iii) thorough the whole year. Highest gut fluid enzymatic activity was found at pH 6 in the foregut. Two main peaks of enzymatic activity during whole year correspond well with the most intensive feeding periods of adult beetles.

Our data are the first to demonstrate the presence of cellulolytic activity in the digestive system (primarily foregut) of *I. typographus,* indicating bark beetles' capability to directly digest cellulose. Furthermore, we propose the endogenous cellulase as the most likely mechanism. Interventions targeting cellulose digestion mechanisms in bark beetles are one of the possible routes to provide a better management of these destructive pests.

Keywords

Cellulase activity, cellulose digestion, gut, *Ips typographus*, spruce bark beetle.

Introduction

Despite of the cellulose being the most plentiful organic material on Earth, and the abundance of xylophagous species, the diversity of species with ability to digest cellulose remains underestimated. However, during the last decade, a growing body of evidence revealed a presence of cellulolytic enzymes in a wide range of invertebrates (Brune, 2014; Oppert et al., 2010; Watanabe and Tokuda, 2001).

The aim of the present study was to demonstrate the presence of cellulase in the spruce bark beetle, *Ips typographus* (L.), (Coleoptera,

Curculionidae, Scolytinae), one of the most economically important xylophagous insect species in European spruce dominated forests, since it causes large scale damage and deforestation over thousands of hectares every year. With regard to the spruce bark beetle in particular, gut cellulase activities have not been previously demonstrated (Chararas, 1979 in Martin, 1983). Identification of the ability to digest cellulose and responsible mechanism may point the way to future interventions oriented to the management of this destructive pest.

Complete enzymatic degradation of cellulose to glucose is accomplished by the 'cellulase complex', a group of cellulolytic enzymes with diverse modes of action, including different activities and substrate specificities (Coughlan and Ljungdahl, 1988). The effective cellulolytic hydrolysis of native cellulose depends upon the concerted and synergistic action of three major types of glycoside hydrolases: (i) endoglucanases (endo-β-1, 4-glucanases); (ii) exoglucanases, which include cellobiohydrolase (exo-β-1, 4-cellobyohydrolases) and exoglucohydrolase (1, 4-β-D-glucan glucohydrolase); (iii) β-glucosidases (Clarke, 1997). First, endoglucanases work by random cleavage of internal glucosidic bonds along a cellulose chain which reduces the degree of polymerization of the cellulose chain into smaller subunits. Second, exoglucanases attack non-reducing or reducing termini of the cellulose chain thereby releasing either cellobiose or glucose. β-Glucosidases complete the third and final step of cellulose hydrolysis by converting the cellobiose and other short cello-oligosaccharides to glucose from the non-reducing end (Breznak and Brune, 1994).

Four mechanisms of cellulose digestion have so far been described in insect. Cleveland (1924) brought the first evidence of the cellulolytic capacity of symbiotic protozoa residing in the hindgut. Complete loss of viability after the defaunation of the hindgut in the eastern subterranean termite *Reticulitermes flavipes* (Rhinotermitidae) suggested close symbiosis between termites and their intestinal protists and have identified the cellulolytic capacity of symbiotic protozoa residing in the hindgut as a primary mechanism of cellulose digestion in this species. Termite endosymbionts are anaerobic protozoa from unique genera of flagellates that are observed only in the guts of lower termites and wood roaches (Brune, 2014). Since then symbiotic cellulolytic hindgut flagellates have

been confirmed in many species of lower termites (Brugerolle and Radek, 2006; Inoue et al., 2000; Veivers et al., 1983). However, other mechanisms of cellulose digestion have been proposed: the cellulolytic capacity of symbiotic bacteria (Bignell, 1977; Cruden and Markovetz, 1979; Griffiths and Cheshire, 1987), the ingestion of fungal cellulases with food (Kukor et al., 1988; Kukor and Martin, 1986; Martin and Martin, 1987; Rouland et al. 1988), and the secretion of endogenous cellulases (McEwen et al., 1980; Scrivenger et al., 1989; Yoke, 1964).

These propositions have been most commonly investigated in termites (Isoptera), which are the most efficient cellulose digesters with assimilation efficiencies of almost 100% (Ohkuma, 2003; Wood, 1978). In the alimentary tract of lower termites, cellulolytic flagellate protozoans may be present, as well as diverse populations of bacteria, and endogenous cellulases (Breznak and Brune, 1994; Ohkuma, 2008). Higher termites lack flagellate protozoa and so cellulose digestion is accomplished by a combination of cellulases secreted by gut cells with bacterial enzymes (Brune, 2014). The sites of expression and secretion of endogenous cellulases in lower termites are salivary glands, whilst in the higher termites; cellulases are secreted by the midgut epithelium (Tokuda et al., 2004). Symbionts (bacteria and/or flagellates) in lower and higher termites dwell in the hindgut (Brune, 2014).

Reports on cellulolytic activity in various species of bark beetles (family Curculionidae) are considerably less abundant than reports on termites. Cellulolytic bacteria have been isolated from the gut of *Dendroctonus rhizophagus* (Morales-Jiménez et al., 2012) and red turpentine beetle *Dendroctonus valens* (Morales-Jiménez et al., 2009). Fungal cellulase has also been confirmed in the xylophagous southern pine beetle *Dendroctonus frontalis* (Valiev et al., 2009). On the other hand, cellulase activity was not detected in bacteria isolated from southern pine beetle *Dendroctonus frontalis* and pine engraver beetle *Ips pini* (Delalibera et al., 2005). The only attempt to detect cellulolytic activity in the spruce bark beetle has been made by Chararas (1979 in Martin, 1983) with negative results.

Given the scarcity and inconsistency of data on bark beetles in general, and the spruce bark beetle in particular, the present study aims to identify and characterize cellulase activity in adult spruce bark beetles: (i) in the whole gut at different pH, (ii) in different regions of the gut (foregut,

midgut, and hindgut) and (iii) fluctuations over the course of one year. Identification of the ability to digest cellulose and responsible mechanism may point the way to future interventions oriented to the management of this destructive pest.

Methods

Insect collection and dissection

Bark beetles were collected in the Šumava National Park, Czech Republic, in 2013 between 600 – 1100 m a. s. l. and dissected (on ice) the same day of collection or stored at 4°C for no more than 24 hours until dissection could be performed. Ringer´s physiological saline was used in the preparation of all extracts and for homogenization.

For experiments guts were dissected as follows: (i) for the detection of cellulase activity at different pH, five pooled samples were used, each comprising of 250 whole guts; (ii) for cellulase activity detection in each region of the gut, eight pooled samples were used each comprising of 50 foreguts, midguts or hindguts; (iii) for the detection of yearly fluctuation of cellulase activity, five pooled samples were used, each comprising of 20 whole guts.

Dissected tissues were sonicated (Ika Labortechnik, Staufen, Germany), and centrifuged at 5000 rpm for 3 min at 4°C (Hettich Lab Technology, Tuttlingen, Germany). Supernatants were then transferred to new tubes, stored at -80°C and used for protein and cellulase detection.

In all experiments, adult bark beetles were used. Insect for the experiment with (i) pH optima and (ii) foregut, midgut and hindgut enzymatic activity was actively feeding on phloem. In the experiment of (iii) cellulase activities over the whole year, bi-monthly collection of insect was used.

Determination of cellulolytic activity

Cellulase activity was measured using EnzChek cellulase fluorescent substrate (Invitrogen detection technologies, Waltham, USA; product number E33953) according to the manufacturer's protocol. In brief, the stock substrate solution was prepared by dissolving 1 mg cellulase substrate in 1.86 ml of 50% DMSO. A working solution was prepared by adding 7.44

ml of digestion buffer to the substrate solution. Test sample (supernatant), standard sample (positive control), or blank, were transferred in triplicates to a 384-well microtiter plate (black, flat bottom) and the working solution was added immediately before fluorescence readings were taken. Fluorescence readings were recorded continuously for 90 minutes. Emission at 452 nm was observed under excitation wavelength of 339 nm in a micro-plate fluorescent reader Infinite 200 (Tecan AG, Maennedorf, Switzerland). A commercial cellulase standard enzyme from *Trichoderma reesei* (product number ATCC 26921) was purchased from Sigma-Aldrich, Saint Louis, USA and prepared according to provided instructions. In all cases Britton-Robinson buffer was used (Briton and Robinson, 1931). Cellulolytic activity in foregut, midgut, and hindgut and detection of enzymatic activity during whole year were tested at the optimal pH 6. The cellulose activity was expresses as the fluorescence change per minute. Each data point is recalculated to cellulolytic activity of one whole gut or gut region.

To provide the same protein content of the gut samples Bicinchoninic Acid Kit was used (Sigma-Aldrich, Saint Louis, USA; product number BCA1) with BSA as standard (Serva Electrophoresis, Heidelberg, Germany; product number 11920).

Design and statistics

To test the effect of pH on cellulase activity a one factor (pH) repeated measures experimental design was used with 11 levels (pH 2 – pH 12). Cellulase activity in different areas of the gut was tested with a one factor (gut region) independent measures experimental design, with three levels (foregut, midgut and hindgut). Cellulase activity monitored over the whole year was tested using a one factor date of the collection experimental design with 23 levels (starting 15th January 2013, and ending 13th December 2013). In all cases cellulase activity measured as fluorescence change per minute was the dependent variable.

Datasets representing cellulase activity in different pH substrates, different gut regions and changes in cellulolytic activity during whole year were each evaluated using one-way analysis of variance (ANOVA) suitable for the individual designs already specified. Tukey's HSD and Least Significant Difference (LSD) test was used for post-hoc comparison. In all

cases, the alpha criterion for rejection of the null hypothesis was set at $p = 0.05$.

Results

pH dependence of cellulose activity

One-way repeated measure ANOVA showed a significant effect of pH on the degradation of substrate for cellulase detection in whole gut ($F_{(10, 30)} = 1429.1$, $p < 0.001$, see Fig. 1). The cellulase exhibited enhanced activity in the mildly acidic pHs (from 4 to 7) with the maximum at pH 6. The activity at pH 6 was significantly higher than activities at all other pHs (post hoc LSD test, maximum $p < 0.001$) except pH 5 ($p=0.06$). The pH profile showed a sharp drop in activity above pH 6 (between pH 6 and pH7). Above pH 7 and below pH 3 enzymatic activity was negligible (Fig. 1).

Differences in cellulolytic activity recorded in foregut, midgut and hindgut

Significant effect of the gut region on the level of cellulolytic activity was observed (One-way ANOVA, $F_{(2, 20)} = 152.86$, $p < 0.001$, see Fig. 2). Post hoc Tukey's HSD tests revealed significantly higher enzymatic activity in the foregut compared to the midgut ($p < 0.001$) and the hindgut ($p < 0.001$).

Differences in cellulolytic activity recorded during the whole year

One-way ANOVA showed a significant effect of date of the collection on cellulolytic activity over the whole year ($F_{(22, 68)} = 25.434$, $p < 0.001$, see Fig. 3). Two peaks in activity were observed, one at the end of May and another at the end of September. The lowest cellulolytic activity was recorded in January; thereafter there was progressive increase until the end of May. Between the first activity peak in the end of May and mid-June a significant drop in activity was recorded (Post hoc Tukey's HSD, $p < 0.001$). From mid-July a stable growth of activity was recorded until the end of September, followed by significant decline in the cellulase activity until the end of the year (Post hoc Tukey's HSD, maximum $p < 0.001$, Tab. 1).

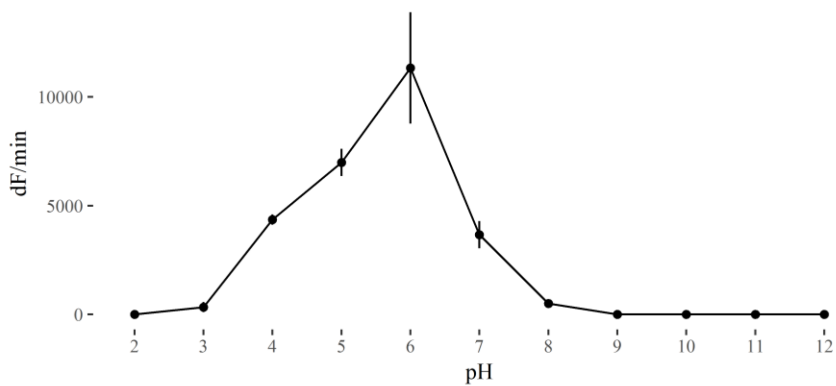

Fig. 1: The pH profile of cellulolytic activity (mean ± S.D.) in the gut of actively feeding adult spruce bark beetles (*Ips typographus*).

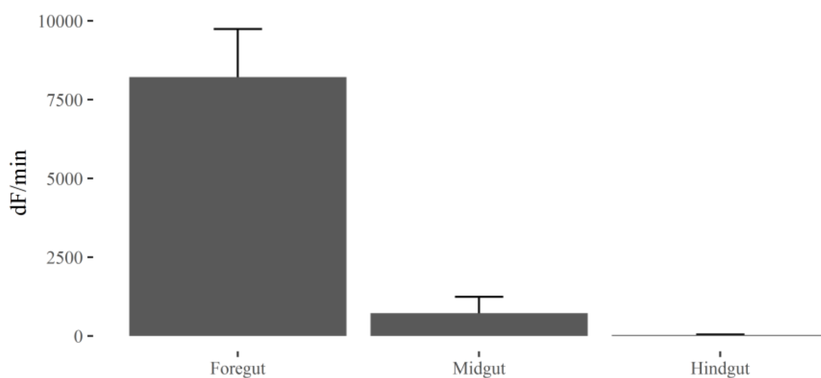

Fig. 2: Cellulolytic activity (mean ± S.D.) in foregut, midgut, and hindgut of actively feeding adult spruce bark beetles (*Ips typographus*).

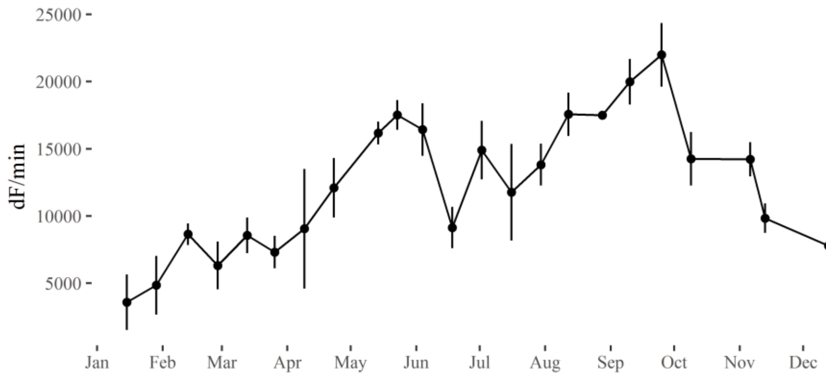

Fig. 3: Yearly profile of cellulolytic gut enzymatic activity (mean ± S.D.) in adult spruce bark beetles (*Ips typographus*).

Discussion

In the present work, we have identified cellulolytic digestion processes in the gut samples obtained from the spruce bark beetle *I. typographus*. The experiment found pH optima for enzymatic activity, varying cellulase activity in different areas of the gut, and fluctuation of enzyme levels during whole year. We are presenting conclusive evidence that *I. typographus* digests cellulose. The cellulase showed a broad range of enzyme activity from pH 4 to 7, with pH 6 as optimum. The highest enzymatic activity was detected in the foregut. Therefore, the foregut is considered the main location of cellulose digestion.

The morphology of the gut may have an impact on digestion and housed symbionts. For this reason, it is important to compare anatomical differences of the gut between termites and bark beetles. In bark beetles, the foregut and midgut dominate in volume, whereas the hindgut is proportionally smaller. Termites have a relatively small foregut and midgut, and always an enlarged and elaborate hindgut with specialized structures where the bulk of symbionts are housed (Watanabe and Tokuda, 2010). These findings indicate that low cellulolytic activity within small hindgut in *I. typographus* imply that cellulose digestion in bark beetles is not realized by microbial or protozoan cellulases and the highest activity in the foregut is likely accomplished by endogenous cellulase. This statement confirms our preliminary results obtained by experiments with treatment of

artificial diet with antibiotics and antimycotics, which had no effect on cellulolytic enzymatic activity. On the contrary, for the termite (*Reticulitermes santonensis*), which harbors a symbiotic community in the hindgut (Bauwens et al. 2013) artificial diet produces significant decrease in enzymatic activity.

The other important factor with possible impact on cellulolytic process is the internal environment (pH) of the gut (Applebaum, 1985). Average pH measured in adults from family Curculionidae is slightly acidic (5.2) in the foregut, nearly neutral (6.9) in the anterior ventriculus, and slightly alkaline (8.4) in the posterior ventriculus (Terra and Ferreira, 1994). Optimal pH of cellulase activity in *I. typographus* is similar to the average pH in other (although not only xylophagous) species of family Curculionidae. Although Curculionidae are an abundant family and measurements of average gut pH are not only from xylophagous species, when we compare their average pH of foregut and pH optima of cellulase from *I. typographus*, there is a tendency that cellulase works nearby in its pH optima.

The next series of experiments evaluated cellulolytic activity during the whole year. Intensity of enzymatic activity corresponds to the life cycle of *I. typographus*; the highest cellulase activity was observed in 23th May and 25th September when adults feed most intensively. First peak of activity in May is likely caused by spring swarming, the highest second peak–in September is likely due to intensive preparation for overwintering when adult bark beetles feed most intensively. Nevertheless, detectable activity occurred even when the bark beetles hibernated and thus had empty guts. This indicates that enzyme release and enzyme activity in gut lumen was not solely influenced by the presence of food.

Conclusion

Our study provides a first description of basic properties of cellulase activity in the spruce bark beetle *I. typographus.* Although the production of cellulase in animals is usually mediated by cellulolytic microorganisms, we provide great evidence, that *I. typographus* secrete endogenous cellulase in the foregut.

It is still not clear how much metabolic energy *I. typographus* can obtain from digestion of cellulose and how it is important for the nutrition, but

because level of enzyme reflects life activity of beetles we may assume that cellulase play an important role in energy metabolism. Moreover, cellulase may be used as target for the design of novel insecticides.

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CHAPTER IV

Biochemical characterization and compartmentalization of the digestive proteases and carbohydrates of the spruce bark beetle, Ips typographus (L.) (Coleoptera: Curculionidae: Scolytinae).

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(Manuscript)

Biochemical characterization and compartmentalization of the digestive proteases and carbohydrates of the spruce bark beetle, *Ips typographus (L.)* (Coleoptera: Curculionidae: Scolytinae).

Abstract

Biochemical characterization and compartmentalization of the digestive enzymes is a crucial knowledge for better understanding of physiology of the *I. typographus*. The profile of digestive enzymes, including proteases and glycosidases was investigated for the first time. This study was primarily focused on determination (i) pH optima of enzymes, (ii) the activity of digestive enzymes in foregut, midgut, and hindgut, and (iii) detection of the activity throughout the year of selected enzymes. Proteolytic activities were present mainly in the midgut with pH optima at the alkaline pH. Activity levels of glycosidases were highest in the foregut and their pH optima were around 5. Results suggest that the anterior part of the gut is the major site of polysaccharide digestion at mildly acidic pH, while midgut is the major site of protein digestion in alkaline pH.

Keywords

Digestion enzymes, glycosidases, gut pH optima, *Ips typographus*, proteinases, spruce bark beetle.

Introduction

I. typographus is a highly destructive major pest of spruce forest in Europe and Asia. In the Czech Republic, the *I. typographus* is not only a significant factor in the damaging of spruce stands, but it also represents a problem that has attracted the attention of the wider public in the wake of ongoing outbreaks in the southern and north-eastern parts of the country. In the present work we identify and characterize digestion in the gut tissue of the *I. typographus.* Despite its economic importance, our knowledge and understanding of the biochemistry of digestion in this beetle remains limited, and papers related to this topic are scarce. Deeper knowledge about biochemical characterization of enzymes and gut function in bark beetles is necessary for a better understanding of physiology in maintaining growth,

development, survival, and reproduction (Oyebanji et al., 2014). It may also play an important role in novel biological insecticidal strategies. For successful management of insect pests through digestive enzyme inhibition, it is crucial to identify the exact type of enzymes presented in the alimentary tract, as inhibitors are extremely selective. Thus, we focused on the proper determination of selected enzymes, including proteases and glycosidases. We aimed to determine their pH profile, localize their highest activity in the gut, and detect all year fluctuation in selected enzymes. For the activity profiling, we used a battery of specific substrates and inhibitors.

Digestive proteolytic enzymes catalyze peptide bonds and release free amino acids from dietary protein thus ensuring a supply of essential nutrients crucial for normal growth and development (Chen, 1966; Nation, 2008). There are four subclasses of endopeptidase (serine, cysteine, aspartic and metallo proteinases): this study is focused on serine proteinase, namely trypsin-like, chymotrypsin-like, and elastase-like, completed by aminopeptidases (exopeptidase), because these proteolytic enzymes are commonly found in the midguts of many insect species.

Glycosidases, enzymes which hydrolyze polysaccharides, are classified according to their substrate specificities. Type of glycosidase is related to the diet (Nation, 2008) and *I. typographus* feed on phloem, which contains lignocellulose with cellulose and hemicellulose as the most abundant constituents (Thompson, 1983). Therefore, we focused on cellulase hydrolyze cellulose (discussed in Chapter III), xylanase hydrolyze hemicellulose, amylase hydrolyze starch, and α- and β -glucosidases. α-glucosidases are responsible for final digestion of starch, β -glucosidases for final digestion of cellulose (Terra and Ferreira 1994; 2005). Digestion of carbohydrates produces a source of energy for insect and saccharides are considered to be a possible substrate for the synthesis of fatty acids (Vodrážka, 1996).

One of vegetal protective strategies against pests is secretion of defensive proteins, which target physiological processes in the insect. The better known protective molecules are proteinase inhibitors (Howe and Jander, 2008) which have potential for developing insect pest control strategies. To bypass negative effects of chemical insecticides (which can be harmful to environment) novel insecticidal approaches are being developed, based mainly on the inhibition of digestion. This approach

offers a more benign insect pest control method, which is safer for non-target, beneficial organism (Andow, 2008). Inhibitors tightly interact with active sites on the target digestive enzyme, thereby blocking or altering access to the substrate (Garcia-Olmedo et al. 1987). Protease inhibitors block proteolysis within cells, organelles, and fluids important for their biochemical and physiological processes. Finally, inhibitors restrict the availability of essential amino acids which leads to decelerated growth rates and increasing mortality. Proteinase inhibitors were found for all four classes of proteinases, most of them belong among serine- and cysteine proteinases (Barrett et al., 1986; Turk and Bode, 1991).

The aim of the present study is the biochemical characterization of digestive enzymes in *I. typographus* in order to gain novel information about digestive physiology and identification of potential inhibitors for enzymatic inhibition in insect pest control programs.

Methods

Insect

All individuals of *I. typographus* were collected from the field in the Šumava National Park, Czech Republic, at 600 - 1100 m a. s. l. Adult bark beetles were used in all experiments. Light adults, actively feeding on phloem were used for the (i) pH optima and (ii) foregut, midgut, and hindgut enzymatic activity experiments. Adults collected bi-monthly were used in the determination of the enzymatic activities over the whole year (iii).

Preparation of the I. typographus gut tissue extract

Bark beetles were dissected on ice the same day of collection or stored at 4°C until dissection could be performed, but for no more than 24 hours. Ringer´s physiological saline was used in the preparation of all extracts and for homogenization. Guts were either homogenized whole or separated into the following regions: foregut, midgut and hindgut. For the detection of (i) pH optima, we used five pooled samples, each comprising of 250 whole guts. Detection of (ii) activity in each region of the gut, we used eight pooled samples, each comprising of 50 foreguts, midguts or hindguts. For the detection of whole year enzymatic activity, we used five pooled samples, each comprising of 20 whole guts. Dissected tissues were

homogenized by using sonicator (Ika Labortechnik, GMBH, Germany) and centrifuged at 5000 rpm for 3 min at 4°C (Remote, Tecan, Austria, software: KIM 2001, Daniel Kittrich). Supernatant was transferred to new tubes, stored at -80°C, and used as the enzyme extract for the protein and enzyme detection.

In all cases, Britton-Robinson buffer was used (Britton and Robinson, 1931). pH profile was assayed over a range of pH values from 2 to 12. Enzymatic activity in each region of the gut, determination of all year activity, and activity of inhibitors was measured in pH optima (i.e., the highest enzymatic activity of the whole gut). Measurement of enzymatic activity was performed at 30°C and was expressed as the fluorescence change per minute. Each data point is recalculated to enzymatic activity of one whole gut or gut region.

To provide the same protein content of the gut samples Bicinchoninic Acid Kit was used (Sigma-Aldrich, Saint Louis, USA; product number BCA1) with BSA as standard (Serva Electrophoresis, Heidelberg, Germany; product number 11920).

Profiling component gut peptidases with substrates and inhibitors

Non-specific protease activity was measured using fluorogenic EnzChek peptidase/protease assay kit obtained from Invitrogen (Invitrogen detection technologies, Waltham, USA; cat. no. E6638). Assays were managed according to the instruction manual. Specific peptidase activities were identified and characterized by hydrolysis of the following AMC-fluorogenic (7-amino-4-methylcoumarin-fluorogenic) substrates: for serine proteinases: Z-Phe-Arg-AMC.HCI for trypsin-like proteinase (incubated with inhibitor E-64 to prevent an interference with activity of cathepsin) (Bachem, Torrance, USA; cat. no. I-1160); Suc-Ala-Ala-Pro-Phe-AMC for chymotrypsin-like proteinase (Bachem, Torrance, USA; cat. no. I-1465); MeOSuc-Ala-Ala-Pro-Val-AMC for elastase-like proteinase (Bachem, Torrance, USA; cat. no. I-1270); for aminopeptidase: H-Leu-AMC for leucine aminopeptidase (Bachem, Torrance, USA; cat. no. I-1240) and Met-AMC for aminopeptidase (Peptanova, Sandhausen, Germany; cat. no. 31479-v).

For measuring enzymatic activity in the presence of peptidase inhibitors, the sample was preincubated for 30 min at room temperature in the assay

buffer in the pH optimum with the inhibitors. Protease enzymatic inhibition used Complete Mini EDTA-free protease inhibitor cocktail (Roche, Basel, Schwitzerland; cat. no. 04693159001) and protease inhibitors set (Roche, Basel, Schwitzerland; cat. no. 11206893001), which included inhibitors: antipain for trypsin and cathepsins, bestatin for aminopeptidases, chymostatin for chymotrypsin, E-64 for cysteine proteinase, leupeptin for serine and cysteine proteinases, pepstatin for aspartate proteinases, phosphoramidon for metalloendopeptidases, pefabloc SC for serine proteinases, EDTA for metallopeptidases, and aprotinin for serine proteinases. Concentrations of protease inhibitors were prepared according to instruction manuals.

Proteolytic activity was continuously measured after addition of substrate in a fluorescence reader (Infinite 200, Tecan AG, Maennedorf, Switzerland) at 360 nm excitation and 465 nm emission wavelengths.

Determination of glycosidases activity

Amylase and xylanase enzymatic activities were determined using fluorogenic substrates. Amylase activity was measured using EnzChek Ultra amylase assay kit (Invitrogen detection technologies, Waltham, USA, cat. no. E33651). Xylanase activity was measured using EnzChek Ultra xylanase assay kit (Invitrogen detection technologies, Waltham, USA, cat. no. E33650). α and β- Glucosidases enzymatic activity were measured using colorimetric assay kit obtained from Medibena (Medibena Life Science & Diagnostic Solutions, Wien, Austria, cat. no.: BA_DAGD-100, BA_DBGD-100 respectively). All assay kits were prepared according to provided instructions.

If a commercial standard was not included in the assay kit, the enzyme was purchased from Sigma-Aldrich (Saint Louis, USA) and prepared according to provided instructions (Tab. 1).

Table 1 – List of enzymes used as commercial standards in assays.

Enzyme	Commercial standards
Non-specific protease	Trypsin from bovine pancreas (Sigma T8003)
Trypsin-like	Trypsin from bovine pancreas (Sigma T8003)
Chymotrypsin-like	α-Chymotrypsin from bovine pancreas (Sigma C4129)
Elastase-like	Elastase from porcine pancreas (Sigma E7885)
Aminopeptidase	Aminopeptidase from *Aeromonas proteolytica* (Sigma A8200)
Leucino-aminopeptidase	Aminopeptidase from *Aeromonas proteolytica* (Sigma A8200)
Amylase	α- Amylase from *Bacillus* sp. (Sigma A6380)
Xylanase	Xylanase (Sigma X2753)
Elastase	Elastase from porcine pancreas (Sigma E7885)

Data analysis

To test the effect of pH on enzymatic activity, a one factor (pH) repeated measures experimental design was used with 11 levels (pH 2 – pH 12). Enzymatic activity in different areas of the gut was tested with a one factor (gut region) independent measures experimental design, with three levels (foregut, midgut and hindgut). Enzymatic activity monitored over the whole year was tested using a one factor (time of the collection) experimental design with 23 levels (starting 15th January 2013, and ending 13th December 2013). Inhibition with specific substrates is expressed as percent inhibition of the positive sample. In all cases, enzymatic activity was the dependent variable (measured as the fluorescence change per minute).

All statistical analyses were conducted using IBM SPSS version 22 (IBM, New York, USA). Data representing enzymatic activity in different pH substrates were analyzed using a one-way repeated measure analysis of variance (ANOVA). Data concerning enzymatic activity in different gut regions and changes in enzymatic activity during whole year were evaluated using an independent one-way analysis of variance (ANOVA),

suitable for the individual designs already specified. Significant main effects of ANOVAs were followed with post-hoc Tukey's HSD tests. In all cases, the alpha criterion for rejection of the null hypothesis was set at $p = 0.05$.

Results

pH dependence on enzymatic activity

One-way repeated measure ANOVAs showed a significant effect of pH on the degradation of substrate for all tested proteases, minimum ($F_{10, 20} = 76.63$, $p < 0.001$, Fig. 1B; 2B), and also for all tested glycosidases, minimum ($F_{10, 20} = 10.8$, $p < 0.001$, Fig. 3B)

All tested proteases exhibited enhanced activity from mild to high alkaline pH. Non-specific protease activity showed continuously increasing activity from pH 5 to 12, with the maximum at pH 12. Serine proteases (included trypsin-like, chymotrypsin-like, and elastase-like enzymes) showed the highest activity in pH 12. Chymotrypsin and elastase enhanced activity continuously with pH, while trypsin had the highest activity in broad alkaline pH range (9 – 12). Aminopeptidase (included aminopeptidase and leucine-aminopeptidases) showed pH optima between pH 7 – 9 for aminopeptidase and pH 8 – 9 for leucine-aminopeptidase, both with maxima at pH 8. All proteases showed significantly less activity at the lower pH values compared to higher pH values (post hoc Tukey's HSD, minimum, $p < 0.001$).

All tested glycosidases exhibited the highest activity at the mildly acidic pH. Amylase showed pH optima between 4 - 6, with maximum at pH 5. Xylanase showed the highest activity between pH 5 and 6. Glucosidases showed pH optima at 6 for α-glucosidase and pH optima between 6 and 7 for β-glucosidase. All glycosidases showed significantly less activity at the higher pH values compared to lower pH values (post hoc Tukey's HSD, minimum, $p < 0.001$).

Effect of inhibitors on proteolytic activity

Non-specific proteolytic activity was inhibited over a range of chemical inhibitors. Chymostatin and prefabricated mix of inhibitors fully inhibited proteolytic activity. For the aprotinin, antipain, pefabloc, leupeptin, and E-

64 were recorded only partial inhibition (from 30% to 62%). Inhibition with phosphoramidon and pepstatin was only negligible (7 – 8%). EDTA and bestatin registered no inhibition (Fig 1C).

Hundred percent inhibition with leupeptin and aprotinin was recorded for trypsin specific substrate. Chymotrypsin activity was fully inhibited only after chymostatin treatment. The elastase activity was partially inhibited with all three tested inhibitors: aprotin, leupeptin, and pefabloc (from 5 to 30%). Both aminopeptidases activities were fully inhibited by bestatin (Fig. 2C).

Proteases and glycosidases activities in foregut, midgut, and hindgut

Significant effect of the gut region on the level of proteases and glycosidases activities were observed (One-way ANOVA, for proteases - minimum $F_{2, 22} = 31.08$, $p < 0.001$, Fig. 1A; 2A; for glycosidases - minimum $F_{2, 22} = 12.24$, $p < 0.001$, Fig. 3A). For all tested proteases post hoc Tukey's HSD tests revealed a significantly higher enzymatic activity in the midgut compared to the foregut (minimum, $p < 0.001$) or the hindgut (minimum, $p < 0.001$). For all tested glycosidases post hoc Tukey's HSD tests revealed a significantly higher enzymatic activity in the foregut compared to the midgut (minimum, $p < 0.005$) or the hindgut (minimum, $p < 0.001$).

Proteases and glycosidases activities during the whole year

One-way ANOVA showed a significant effect of date of the collection on non-specific protease, trypsin, and chymotrypsin activity over the whole year (minimum, $F_{22, 86} = 25.20$, $p < 0.001$, see Fig. 4); also there was a significant effect of date of the collection on amylase and xylanase activity (minimum, $F_{22, 86} = 12.92$, $p < 0.001$, see Fig. 4).

For all three tested proteases, there were recorded zero activities until the beginning of April, thereafter we registered a progressive increase in enzymatic activities. For non-specific protease, there were two peaks in activity, first in mid-May and second in mid-August, followed by decline in activity until end of the year. For chymotrypsin, first activity peak was registered in mid-May and second peak in mid-August, similarly.to pattern in non-specific protease. Highest trypsin activity was observed from mid-May to the end of September.

For amylase and xylanase, the lowest activities were observed at the beginning of the year until the end of April. Thereafter a progressive increase leading to two activity peaks. For xylanase the first peak was at the end of May and for amylase in the beginning of June. A second peak is observed in the end of September for both.

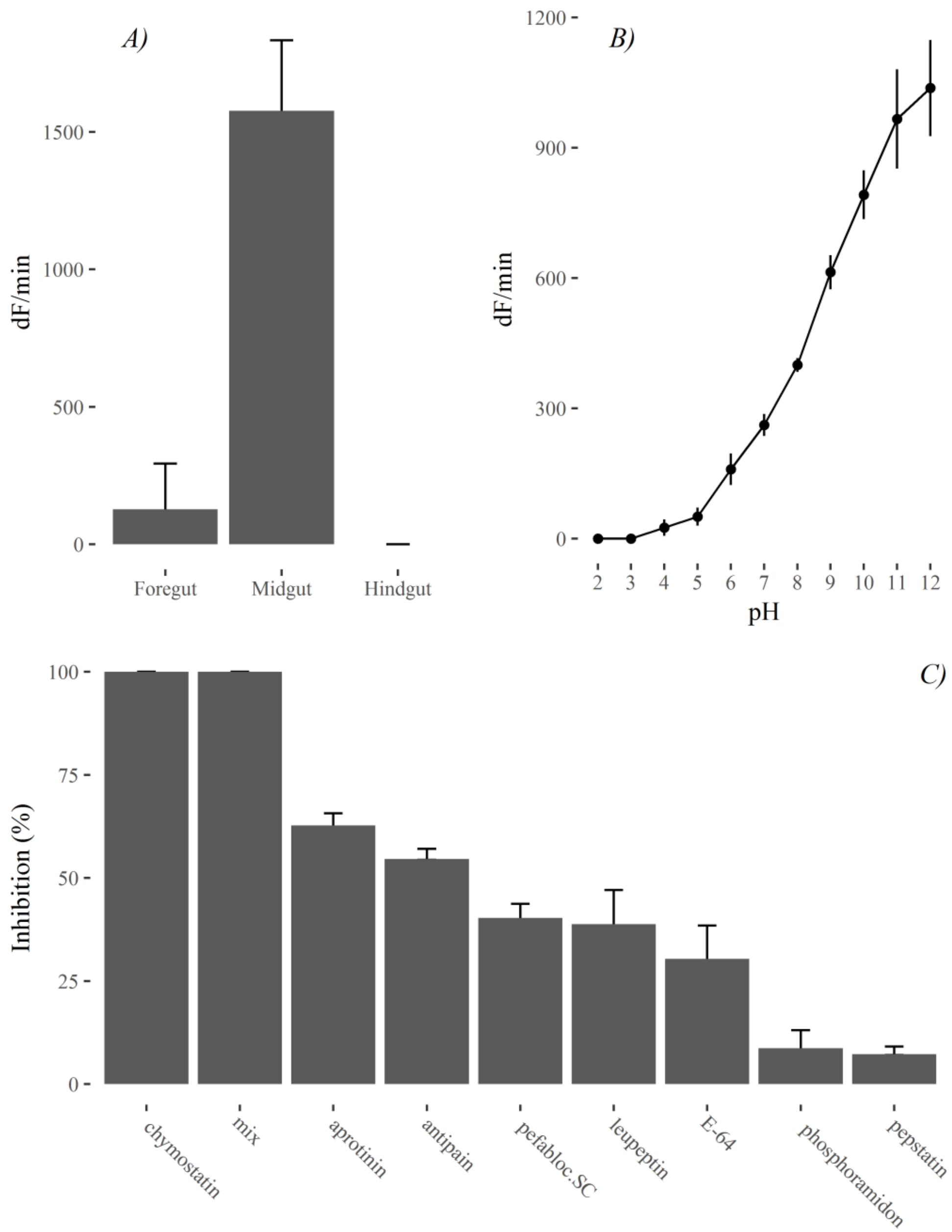

Fig. 1: Non-specific proteolytic activity in the gut of actively feeding adult *Ips typographus*; a) activity in foregut, midgut, and hindgut; b) pH profile; c) inhibitory profiles, (mean ± S.D.).

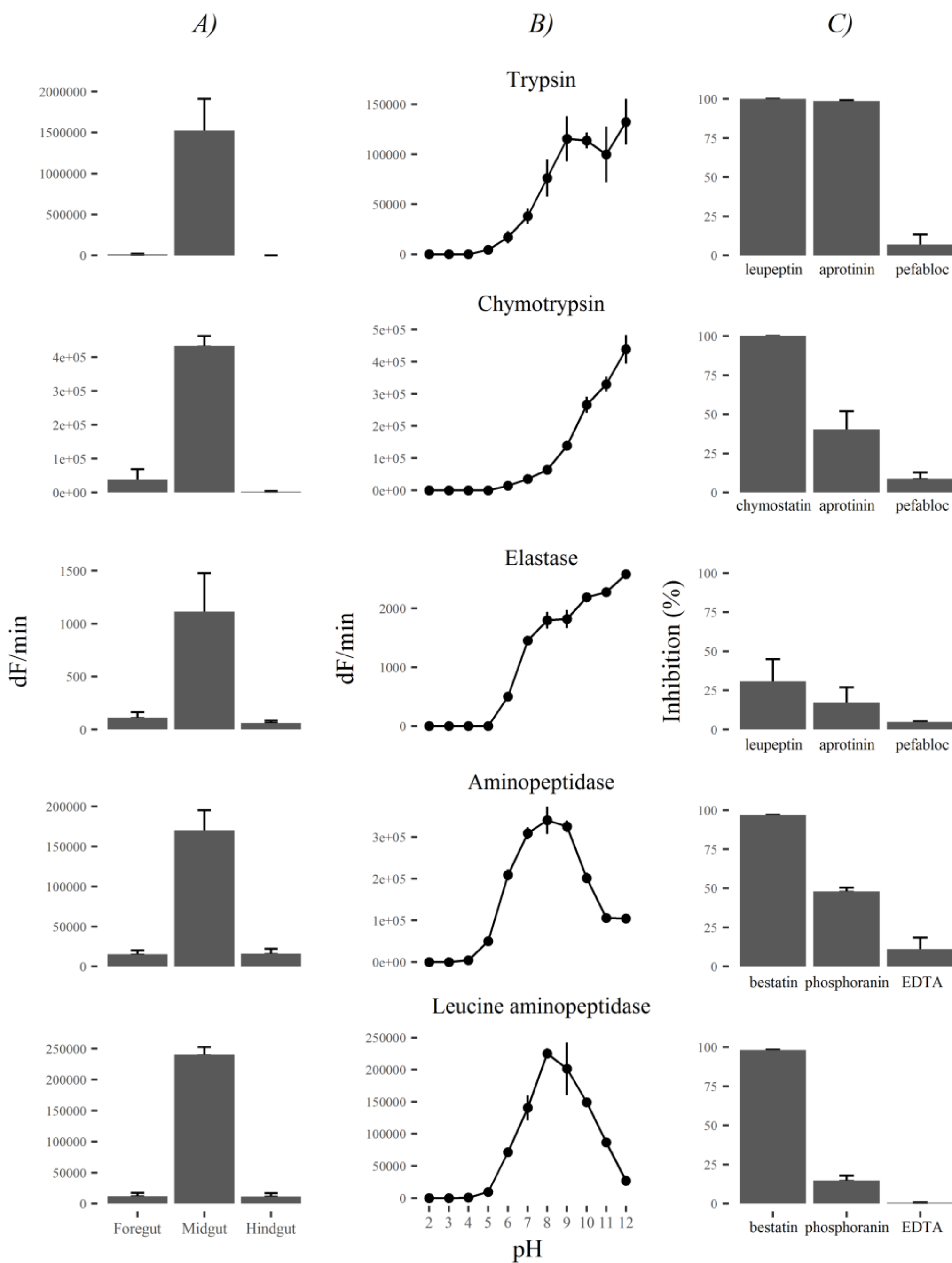

Fig. 2: Proteolytic activity (mean ± S.D.) of individual proteinases in the gut of actively feeding adult *I. typographus*; A) in foregut, midgut, and hindgut; B) pH profiles; C) inhibitory profiles, (mean ± S.D.).

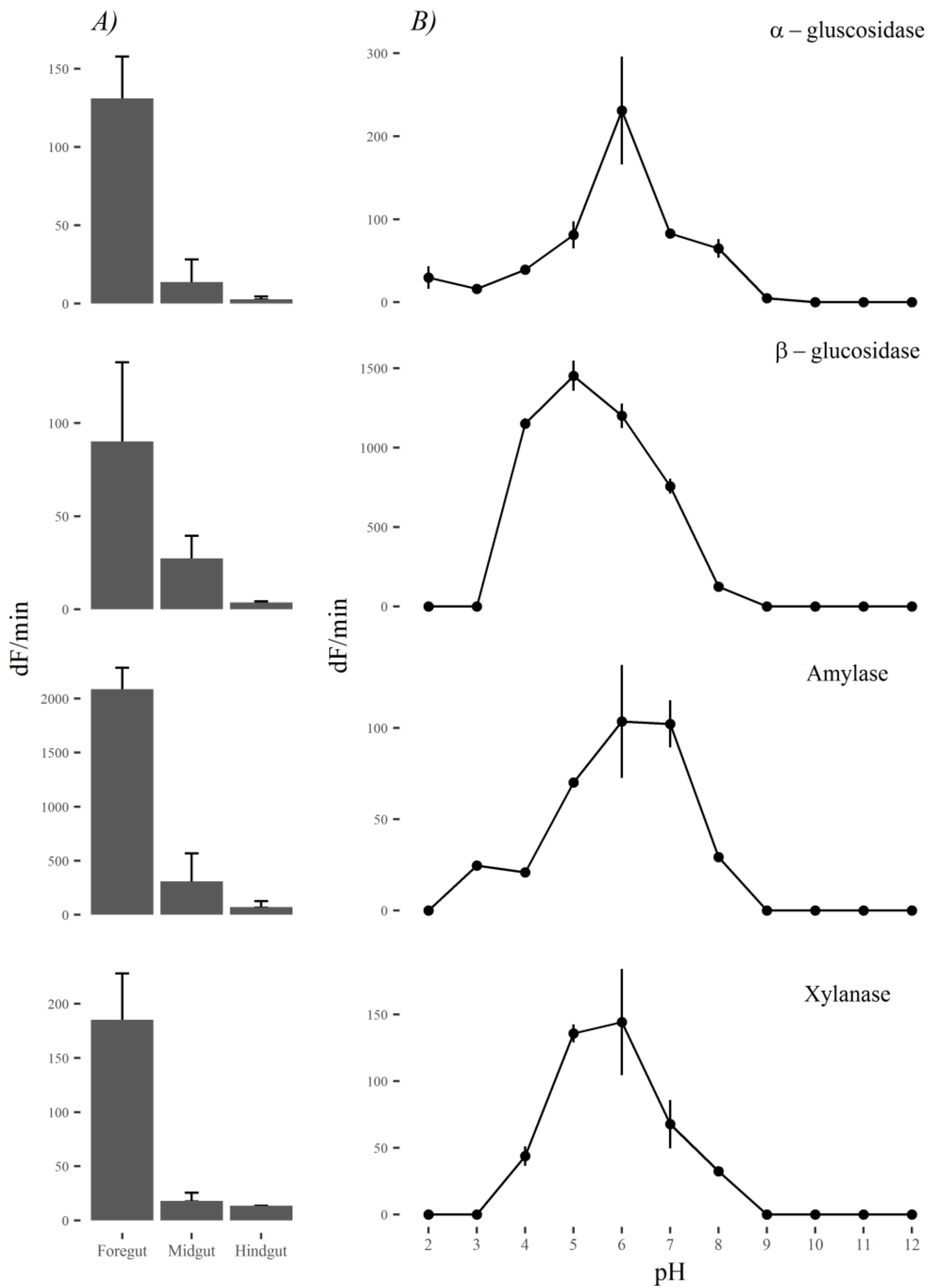

Fig. 3: Enzymatic activity of individual glycosidases in the gut of actively feeding adult *I. typographus*; A) in foregut, midgut, and hindgut; B) pH profiles, (mean ± S.D.).

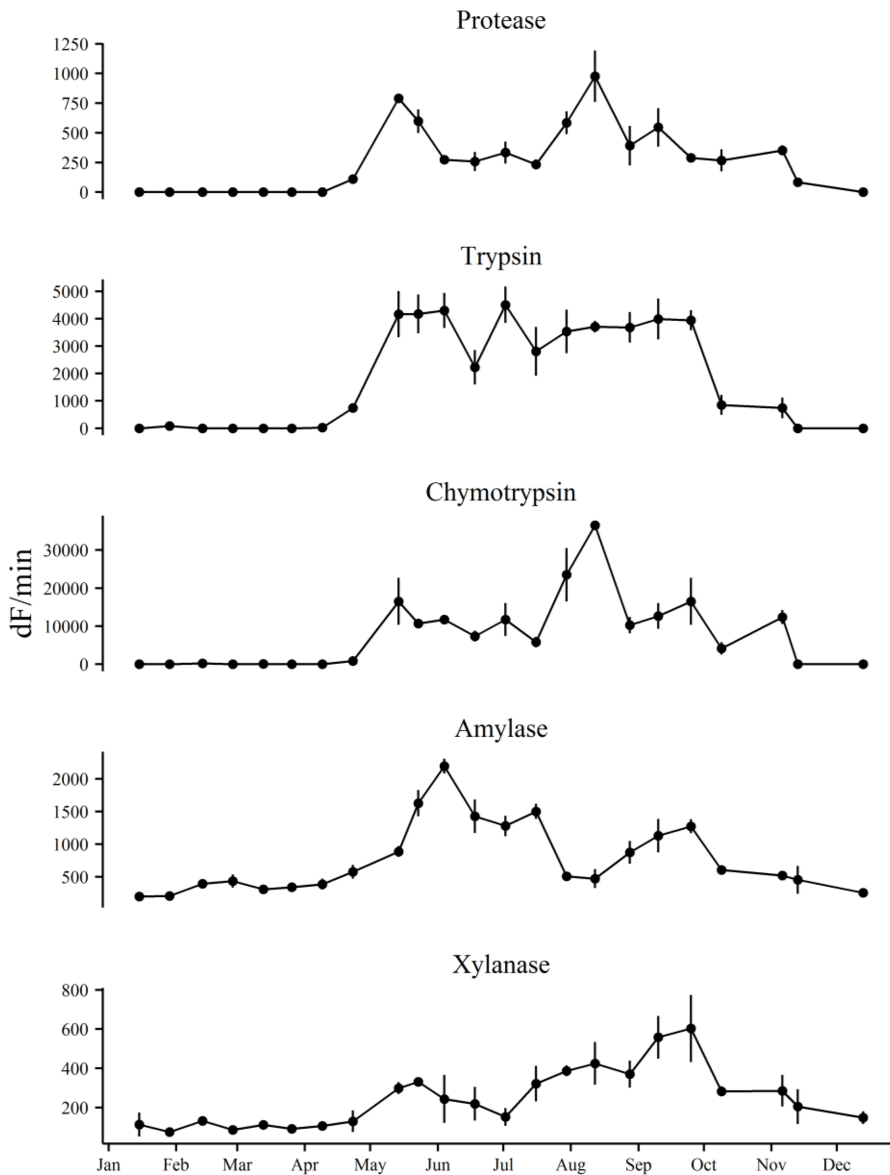

Fig. 4: Annual activity profiles in *I. typographus* for non-specific proteolytic activity, trypsin-like, chymotrypsin-like, amylase, and xylanase activity, (mean ± S.D.).

Discussion

Enzymatic activity and pH optimum

Our data provide evidence that non-specific proteolytic activity in the alimentary tract of fully fed active adults is present from neutral to high alkaline conditions. A broad range of alkaline pH of non-specific proteolytic activity, together with inhibition caused by a wide range of specific inhibitors, suggest utilization of multiple enzymes in bark beetle's protein digestion.

The observation of highest non-specific proteolytic activity in high alkaline pH conditions is in agreement with majority of studies, where presence of serine proteinases suggest proteolytic activity in high alkaline pH (reviewed in Terra and Ferreira, 2012). In our study, activity of trypsin-like, chymotrypsin-like, and elastase-like enzymes was identified by the hydrolysis of specific substrates and their partial or total inhibition by specific inhibitors. Moreover, presence of the second protease group (aminopeptidases) was also detected.

While activity curves of all three serine proteinases more or less copied curve of non-specific activity in broad range of pH (with the highest activity in pH 12), aminopeptidases manifest optimal activity in mildly alkaline pH and their enzymatic activity steeply decreased in highly alkaline pH. This suggest non-equal activity of aminopeptidases in comparison to serine proteinases. If aminopeptidases were equally active as serine proteinases, a second peak would occur in non-specific activity around neutral pH (Fig 2B).

Our results are with agreement with literature, where serine proteinases were found to be active in general in alkaline pH (Nation, 2008), and aminopeptidase with pH from neutral to mildly acid (Cristofoletti et al., 2006).

We therefore suggest that serine proteinases are responsible for most of the proteolytic activity in the midgut of *I. typographus*, phenomenon described in other species (Colebatch et al., 2001; Mahdavi et al., 2013).

Serine proteinases have been identified in extracts from the digestive tracts of insect species across many families (Macedo and Freire, 2011). Trypsin is widely distributed in the majority of insect (Terra and Ferreira, 1994) only with some known exceptions: firstly, in hemipteran species;

secondly in taxa belonging to the series Cucujiformia of Coleoptera like Curculionidae, where trypsin activity was not found at all (Terra and Ferreira, 2012). Chymotrypsin activity among insect taxa is similar to trypsin activity (Terra amd Ferreira, 1994). Also elastase and aminopeptidases have been characterized in many insect taxa (Terra and Ferreira, 2012). Aminopeptidases are described in at least six insect orders: (Orthoptera, Hemiptera, Coleoptera Adephaga, Coleoptera Polyphaga, Diptera and Lepidoptera (Adang, 2013).

We confirmed high activity of amylase, xylanase, and α- and β-glucosidases in the digestive system of fully fed adult bark beetle. We also discovered that pH optima for all tested glycosidases lay in mildly acid conditions. Glycosidases are found in large numbers of insect species, xylanase is studied in detail especially in termites (Matoub and Rouland, 1995; Odelson and Breznak, 1985; Rouland et al., 1988), amylase is characterized in many orders, for example: Hymenoptera, Coleoptera, Diptera, and Lepidoptera (Terra and Ferreira, 1994).

The pH condition in the gut may play an important role in digestion and affect enzymatic activity. The average pH measured in adults from family Curculionidae is slightly acidic (5.2) in the foregut, nearly neutral (6.9) in the anterior ventriculus, and slightly alkaline (8.4) in the posterior ventriculus (Terra and Ferreira, 1994). Comparison of average pH in the gut of the family Curculionidae with pH optima of tested enzymes in *I. typographus* revealed that optimal pH of glycosidases and aminopeptidases is similar to the average pH of species of family Curculionidae (although not only xylophagous), while optimal pH of all tested serine proteinases is dissimilar to the average pH of the gut of the family Curculionidae.

Effect of inhibitors on proteolytic activity

A broad spectrum of specific inhibitors of non-specific proteolytic activity revealed not only the presence of serine proteinases and aminopeptidases, but also the presence of cysteine proteinase (inhibition with papain, leupeptin, and E-64). Aspartate proteinase (inhibition of pepstatin) and metalloproteinase (inhibition of phosphoramidon) is either absent or present in negligible amount. This is consistent with previous literature: cysteine proteinase was found in Hemiptera Heteroptera and in

species belonging to the series Cucujiformia of Coleoptera (Houseman and Downe, 1980; Terra and Ferreira, 1994). On the other hand, cysteine proteinase was not confirmed in cucujiform cerambycid beetles (Johnson and Rabosky, 2000). The presumption that aspartic proteinase occurs together with cysteine proteinases in Hemiptera and in most Coleoptera order (Terra and Ferreira, 1994) was not confirmed in *I. typographus* but this topic remains unexplored and needs detailed study utilizing highly specific substrates. Metalloproteinase is not widespread in insect (Nation, 2008) and was not confirmed in *I. typographus*.

Although bestatin, inhibitor aminopeptidases, is not effective against non-specific protease activity, in specific substrate for aminopeptidases it exhibited 100% inhibition. The most likely explanation for this contradictory findings is the testing condition for inhibition at pH 12 (optimum for non-specific proteolytic activity), which may not be ideal for specific inhibitor.

Proteases and glycosidases activities in foregut, midgut, and hindgut

In all cases, the highest activity of proteases was detected in the midgut, while the highest activity of glycosidases was detected in the foregut. These results suggest that the anterior part of the gut is a major site of polysaccharide digestion, while midgut is the major site of protein digestion. Our results are with agreement with literature, where proteolytic activity in insect has been observed mainly in midgut (Schumaker et al., 1992; Terra and Ferreira, 2012). However, we observed glycosidase activity in *I. typographus* predominantly in foregut, while glycosidases in insect predominantly occur in salivary gland or midgut (Nation, 2008; Schumaker et al., 1992).

Proteases and glycosidases activities during the whole year

The next series of experiments evaluated enzymatic activity during the whole year. We tested activity of selected glycosidases (amylase and xylanase) and proteases (non-specific, trypsin-like, and chymotrypsin-like protease).

Annual activity curves for all measured enzymes more or less copied the life cycle of *I. typographus.* In all tested proteases we have seen zero activity till April, while in glysocidases, minimal activity was detected even

when the bark beetles hibernated and thus had empty guts. This indicates that glycosidases are being released even during hibernation and glycosidases activity in gut lumen is not solely influenced by the presence of food. In addition, metabolized saccharides may be a possible substrate for the synthesis of fat (Vodrážka, 1996). Increased enzymatic activity in all tested enzymes corresponded with spring activity of *I. typographus*. A second peak in activity in glycosidases was recorded at the end of September, while in proteases second activity peak occurred in mid of August which may be attributed to intensive preparation for overwintering. After this date, enzymatic activity progressively decreased to the end of the year.

To conclude, we are first to confirm presence of serine and aminopeptidases proteinases and high probability of presence of cysteine proteinase in midgut of *I. typographus*. Moreover, we are first to bring evidence about activity of amylase, xylanase, and α- and β- glucosidases in the foregut of adult bark beetles. For proteolytic enzymes and glycosidases, we were able to identify pH optima and curve shapes of annual enzymatic activity.

Acknowledgments

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CHAPTER V

Conclusion and future perspective

In the present work, we focused on overwintering success of unfinished generations in the Central European population of the spruce bark beetle, *I. typographus.* We identified an enzymatic system functioning in the digestive gut tissue of *I. typographus*. The main findings and implications of the thesis (based on three manuscripts) is summarized below:

I. Our results indicate that in Central Europe immature stages of *I. typographus,* especially pupae, can significantly contribute to spring swarming, and thus the role of newly hatched beetles cannot be neglected. According to current legislation, infested trees that are cut after the end of vegetation season (October, November) and contain predominantly subadult bark beetles, can be removed as late as following spring, and are not considered dangerous due to high winter mortality of larvae and pupae. Our results indicate that above-mentioned strategy should be modified and all infested timber removed as soon as possible.

II. We demonstrated, for the first time, that *I. typographus* is able to digest cellulose. The highest enzymatic activity of cellulase was detected in the foregut at pH 6 as optimum. Although the production of cellulase in animals is most often mediated by cellulolytic microorganisms, we provide strong evidence, that *I. typographus* secrete endogenous cellulase in the foregut. This finding has implications for potential pest control techniques that might target cellulase activity and therefore disrupt nutrition in the spruce bark beetle.

III. We demonstrate presence of proteolytic enzymes (especially serine proteinase) and glycosidases in the alimentary track of *I. typographus*. Activity of proteolytic enzymes was highest in the midgut from slight to high alkaline pH. Glycosidases activity was highest in the foregut with mildly acidic pH as optimal. It follows that the anterior part of the gut is the major site of polysaccharide digestion at mildly acidic pH optima, while midgut is the major site of protein digestion in alkaline pH optima. Better understanding of the digestive physiology may have implications for developing new biological insecticidal strategies through digestive enzyme inhibitors.

The European spruce bark beetle, *I. typographus*, is highly destructive insect pest and it is a significant factor in the damaging of spruce stands; yet we still know little about its overwintering capacity and digestive biochemistry. Better insight into over-wintering potential and detailed understanding of digestion would help us to better protect our forests and reduce the economic and environmental costs that result from this damaging pest.

APPENDIX

CURRICULUM VITAE

Date of Birth: November 16th, 1984
Nationality: Czech

EDUCATION

Ph.D. student in Physiology and Immunology — Since 2010
University of South Bohemia, Faculty of Science

Mgr. in Teacher of Biology for secondary schools — 2011 – 2015
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Mgr. in Clinical Biology — 2008 – 2010
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Bc. in Biology — 2005 – 2008
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WORK EXPERIENCE: ACADEMIC POSITIONS

Junior researcher — Since 2014
National Institute of Mental Health, Prague

Research and Teaching assistant — 2012 – 2014
University of South Bohemia, Faculty of Science

Junior researcher — 2010 – 2014
Biology Centre of the ASCR, Institute of Entomology

INTERNATIONAL CONFERENCES

IV International Conference on Novel Psychoactive Substances (2016)
Budapest, Hungary

Joint Meeting - European Behavioral Pharmacology Society (2015)
Verona, Italy

IUFRO World Congress - Sustaining Forest, Sustaining People: The Role of Research (2014) *Salt Lake City, Utah*

2nd Annual Meeting of the European PhD Network in Insect Science (2011) *Tours, France*

IUFRO WP.7.03.05 – Ecology and Management of Bark and Wood Boring Insect (2011) *Sopron, Hungary*

Fourth Workshop on Genetics of Bark Beetles and Associated Microorganisms (2011) *Sopron, Hungary*

10th IUFRO workshop of WP 7.03.10 – Biotic Risks and Climate Change in Forest (2010) *Freiburg, Germany*

TEACHING EXPERIENCE

Teaching Assistant in practical course of "Physiology and Developmental Biology" (Faculty of Science, University of South Bohemia, CZ).

COURSES

Certificate of competency according to §17 of the ACT No 246/1992 coll. on Protection Animals against Cruelty in present statues at large. 2015
Charles University in Prague, Central Commission for Animal Welfare (CCAW).

Accredited course of special medical and laboratory methods. 2012
Institute for postgraduate medical education.

INTERNSHIPS

School of Forest Resources and Conservation (3 months, 2012)
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