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Ph.D. thesis

SELECTED ASPECTS OF IMAGINAL DIAPAUSE IN BEETLES (COLEOPTERA)

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Annotation

The thesis is focused on the role of photoperiod and temperature upon the course of imaginal diapause in two important pest species *Ips typographus* (L.) and *Leptinotarsa decemlineata* (Say.). The changes in physiology of both species were studied and a simple method for discriminating diapausing and active adults was proposed.

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- Doležal P., Bextine R.B., Doležalová R., Miller T.A.** (2004): Novel methods of monitoring the feeding behavior in the glassy-winged sharpshooter (*Homalodisca coagulata*). *Ann. Entomol. Soc. Am.* 97 (5): 1055 – 1062.
- Habušová O., Turanli F., Spitzer L., R ž i ka V., Doležal P., Sehnal F.** (2006): Communities of beetles and spiders in the stands of normal and genetically modified maize. *Pesticides 2005*: 125 -131.
- Habušová O., Turanli F., Doležal P., R ž i ka V., Spitzer L., Hussein H.M.** (2006): Environmental impact of Bt maize – three year experience. *IOBS/WPRS Bulletin*: 57 - 63.
- Doležal P., Sehnal F.** (2007): Effects of photoperiod and temperature on the development and diapause of the bark beetle *Ips typographus*. *J. Appl. Entomol.* 131(3): 165 – 173.
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- Zelený J., Doležal P.** (2000): Some bark beetles (Scolytidae, Coleoptera) and their bioregulators on spruce in the Šumava National Park . In: V. Podhrázský, H. Ryšánková, S. Vacek, I. Ulrichová (eds.) Monitoring, research and management of ecosystems in the Šumava National Park , November 27-28, 2000, Kostelec nad ěrnými lesy, Czech Republic. pp. 61-63.

- Doležal P., Sehnal F.** (2002): Diapause in the spruce bark beetle (*Ips typographus*(L.)), 2nd. Czech – Japanese seminar on entomology – Insect Photoperiodism and Rhythmicity, August 5 – 8, eské Bud jovice.
- Doležal P., Sehnal F.** (2002): Diapause in the spruce bark beetle (*Ips typographus*(L.)), IUFRO WP 7.03.06, 7.03.07, 7.03.10 „Methodology of Forest Insect and Disease Survey in Central Europe, September 1 – 6, Krakow, Poland.
- Zelený J., Doležal P.** (2004): Bark beetles (Scolytidae, Coleoptera) on spruce in the Bohemian Forest. In: Dvo ák L., Šustr P (Eds): Aktuality šumavského výzkumu II. Book of proceedings, October 4-7, 2004, Srní, Czech Republic: 221-223.
- Doležal P., Sehnal F.** (2005): Diet alters the profile of digestive proteases in the caterpillars of *Spodoptera littoralis*, IVth International Conference on Arthropods “Chemical, Physiological And Environmental Aspects”, University Of Wroclaw, Faculty Of Chemistry, Zakopane, September 18-23, 2005.
- Doležal P., Sehnal F.** (2005): Exposure to low temperature accelerates termination of imaginal diapause in the bark beetle *Ips typographus* from temperate climatic zone, Taiwan – Japan – Czech Republic Cooperation Symposium on Entomology, Taipei, Taiwan, April 18 – 21, 2005.
- Habuřtová O., Turanlı F., Hussein H., Doležal P.** (2005): Changes in entomofauna in Bt maize field during a period of three years, In Proceedings of IOBC/WPRS Working Group on GMOs in Integrated Plant Production “Ecological Impact of Genetically Modified Organisms”, Lleida, Catalonia, Spain, June 1 – 3, 2005.
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INTRODUCTION

The Class of Insecta represents more than 90 percents of animal species known to science. The fossil records indicate that first wingless insects appeared on the Earth about 400 million years ago. Since Devonian Period the insects evolved many powerful strategies that contributed to enormous evolutionary success of the Class. The flight ability facilitated rapid worldwide expansion, diversification of the larval and adult stages enabled occupation of different habitats, and the co-evolution with plants provided new food resources. Last but not least, the insects developed mechanisms to percept signals preceding seasonal changes of the environment and adapted their life cycles to unfavorable conditions. More than 70 years of intensive ecological, behavioral, physiological, metabolic and recently also molecular research yielded considerable knowledge of the effects of both biotic and abiotic factors on insect life cycles. Primary classification of insects' responses to environmental changes is based on the predictability and duration of the response (**Tab. 1**).

Adverse conditions in either biotic or abiotic factors lead to a suppression of development called dormancy that is further subdivided into the following types. Sudden disturbances such as rapid temperature variations have a direct effect on general metabolism and may cause behavioral adjustments (escape movement, body torpor, etc.) that are controlled by the neural system. States of immediate metabolic suppression and developmental arrest are called quiescence. Survival through the long-lasting cyclic changes, e.g. seasonal fluctuations in the temperature and humidity in the temperate climatic zone, is usually associated with the genetically based and neurohormonally mediated physiological alterations collectively termed diapause. In some species, diapause has become integrated into the life cycle and occurs in every generation without an external signal; such cases are classified as obligatory diapause. However, most insects enter a facultative diapause that is induced by outer signals and its timing is therefore well adjusted to certain seasonal time. Diapause occurs at a specific developmental stage(s) that are characteristic for each species and its genotypes.

Last three decades of research proved that diapause is not just a constant and passive state of low metabolic activity and suspended development or reproduction. From the recent

point of view, diapause is a dynamic process, during which insect organism and its responsiveness to various cues undergo changes (Tauber et al., 1986; Danks, 1987; Košál, 2006). Diapause can be divided into three main phases 1) Pre-diapause; 2) Diapause; and 3) Post-diapause. Each of them is probably composed of subphases (**Fig 1**) but no consensus regarding subphasic classification and terminology has been reached (reviewed by Košál, 2006). The following text considers only the main phases of diapause development.

Tab. 1 – Classification of environmental changes and insect responses (modification of original table published by Tauber et al., 1986)

Duration	Predictability	
	Unpredictable	Predictable
Short-term change	Sudden temperature changes Expended food sources of current habitat Adaptations: Aseasonal quiescence, migration and polyphenism	Diurnal and lunar cycles in temperature, humidity, food sources, etc. Adaptations: Diurnal and lunar rhythms of physiological and behavioral processes
Long-term change	Drought Habitat modification (urbanization, logging, agriculture) Windthrow disasters Adaptations: Aseasonal quiescence, genetic polymorphism, evolutionary change	Seasonal cycles in temperature, humidity, food sources etc. Adaptations: Dormancy, seasonal migration and polyphenism

The pre-diapause phase is characterized by ongoing development although signals indicating imminent changes are perceived and stored to reroute ontogenesis to a diapause programme. These signals may be both direct and indirect. Indirect environmental cues are also termed token stimuli (Lees, 1955), because they are not unfavourable by themselves but signal an advent of adverse seasonal changes. These mechanisms assure that diapause is induced before the environment becomes hostile. Photoperiod, i.e. the ratio of daylight to the night darkness, is the most common and the best studied token stimulus. Specific receptors inside the brain and compound eyes (reviewed by Numata et al., 1997) enable insects not only to record the daylength, but also the number of days shorter than a diapause-inducing threshold (reviewed by Denlinger, 2002). Presence of specific chemicals in the diet is another example of a token stimulus. Although their role as inducing signals is mostly known from the tropics and other geographic areas, where changes in the daylength are

insignificant (Denlinger, 1986), chemical substances expressed by host plants may act as strong signals also in the temperate zone. Ushatinskaya (1981) demonstrated their role in *Leptinotarsa decemlineata* (Say) and Führer (1981) in *Ips typographus* (L.).

The perception of inducing signal and the initiation of diapause may take place in the same developmental stage or be separated by a period of time during which ontogenesis continues, the insects store energy reserves, and migrate to habitats suitable to survive adverse conditions that are to come. This preparation phase for diapause may span two generations in some insect species (reviewed by Danks, 1987; Mousseau and Dingle, 1991).

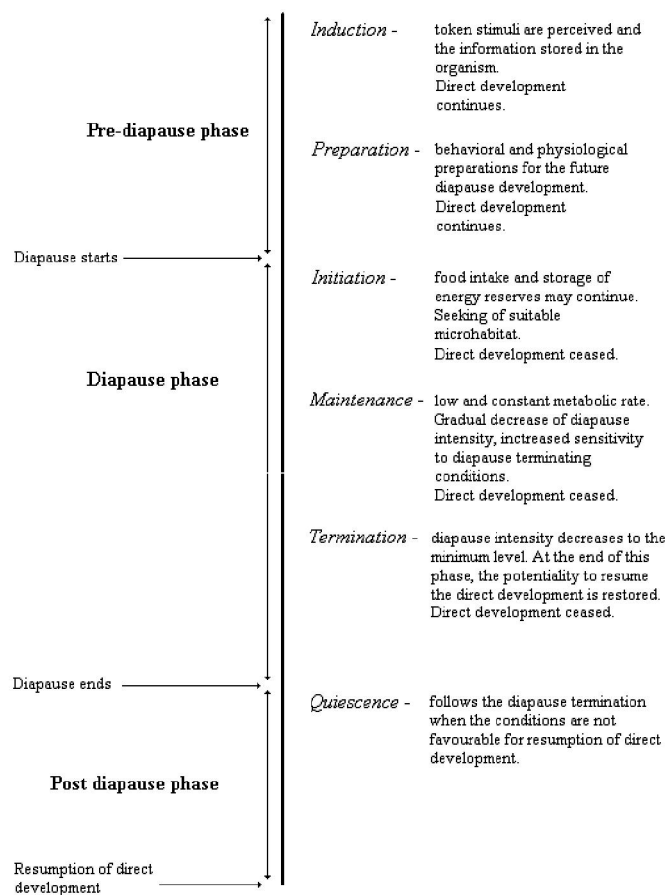


Fig 1 – The phases and subphases of insect diapause as defined in Košál (2006).

The diapause phase is characterized by suppressed metabolism associated with reduced feeding and manifested by a low rate of oxygen consumption (first recorded by Schneiderman and Williams, 1953). Once induced, the intensity of diapause increases under

the diapause-inducing conditions and the pre-diapause physiological and behavioral preparations for adverse conditions may continue. Development is usually arrested as soon as the pre-determined diapause stage has been reached. Diapausing insects usually remain sensitive to token stimuli that intensify diapause and prevent precocious restoration of development. The metabolic rate becomes stabilized at a low level. The onset of diapause phase may be marked by morphological and physiological changes. For examples, the diapausing larvae of the stonefly *Taeniopteryx burki* lose abdominal cerci (Harper and Hynes, 1970) and diapausing adults of various species, including *Leptinotarsa decemlineata* degenerate the flight muscles (Wolda and Denlinger, 1984). However, such symptoms are not always present and decreased metabolic rate therefore remains the most reliable and general diapause indicator.

Diapause phase is most commonly terminated following perception of specific environmental signals such as low temperature, change of the photoperiod, or increased humidity (Tauber et al., 1986). However, examples of a spontaneous diapause termination (Danks, 1987; Musolin and Saulich, 1996) as well as diapause extension for several years (Tauber and Tauber, 2002), have also been recorded. The physiological mechanisms of such processes remain unclear. Once the diapause phase is terminated, the insects may resume development providing that environmental conditions are permissive. If they remain hostile, the insects persist in so called post-diapause phase until a change of the limiting factor occurs. Many species from the temperate zone are good examples of such post-diapausing insects. Their diapause is terminated up to several months before temperature conditions permit development (Tauber and Tauber, 1976; Tauber et al., 1986). Although the post-diapause insects are capable to resume development, some diapause features, e.g. enlarged fat body or high levels of cryoprotectants, persist to ensure survival at the currently low temperature.

Economic interests fostered research on diapause in various pests that became models for other insects. Since the development and reproduction cease during diapause, detail knowledge of factors that regulate diapause may open way to novel methods of pest management. My PhD work is a contribution to the knowledge of the induction, course, and termination of reproductive diapause in beetles.

Two species from different families of beetles (Coleoptera) were chosen as experimental objects.. Both of them, the Colorado potato beetle, *Leptinotarsa decemlineata* (Say.) (Coleoptera; Chrysomelidae) and the spruce bark beetle, *Ips typographus* (L.) (Coleoptera; Curculionidae), may enter facultative imaginal diapause. The diapause of *Leptinotarsa decemlineata* has been a target of numerous studies since 1950s, starting with classical papers of de Wilde, (1955, 1959, 1961, and many others) and continuing with articles of his students on the hormonal control of diapause (reviewed in de Kort, 1990). The life cycle of the Colorado potato beetle (CPB) can be summarized as follows. The adults of CPB leave their overwintering habitats during May or early June, search for the host plants, mate (females can also be fertilized before entering diapause), and start to lay eggs within two days after mating. One female lays up to 300 eggs per one ovarian cycle. First instar larvae hatch after 1 – 4 days, depending on the air temperature and humidity, and feed on leaves of the host plant. Larval development proceeds in four instars and lasts 15 – 20 days. The post-feeding larvae of the fourth instar dig into the soil and undergo larval-pupal moult. The pupal stage lasts up to 8 days and newly ecdysed adults start to reproduce within one week after their emergence from the soil. Exposure to photoperiod shortening in late summer and autumn induces imaginal diapause. Although short photoperiod is the main inducing factor (de Wilde, 1955, 1965), the food quality (Ushatinskaya, 1981) and temperature (de Wilde, 1959) may considerably modify the photoperiodic response. The survival of diapausing adults overwintering 30 – 45 cm deep in a dry soil amounts up to 60%. Increased soil humidity (more than 1.5%) causes high mortality rates (Constanzo et al., 1997). Diapausing beetles have empty guts, their reproductive organs are undeveloped and the flight muscles are atrophied (de Wilde, 1961). Distinct changes are noticeable in the fat body structure (Dortland and Esch, 1970), in the respiration rate and heartbeat frequency. Diapause is terminated by a rise of soil temperature. The major hormonal factor causing diapause termination is an increase in juvenile hormone concentration in the haemolymph (de Kort, 1990).

The spruce bark beetle, *Ips typographus*, is considered a serious pest of spruce monocultures in most European countries. Its massive outbreaks after the World War II stimulated extensive ecological studies (Vité, 1952; Annala, 1969; Wermelinger and Seifert, 1998; Wermelinger, 2004; Zumr, 1985, 1995). However, very few authors dealt with its

diapause and physiology (Führer and Chen, 1979; Schopf, 1985, 1989; Krausseopatz et al., 1995) and our knowledge is fragmentary. The annual cycle of this beetle usually starts at the end of April or during May by the emergence of overwintering generation from the hides under the bark of spruce trees.. Male beetles attack suitable host trees and bore mating chambers in the floem. Females, allured by the male pheromone, allocate the infested trees, mate, and start to oviposit into small holes on the sides of the maternal tunnels. Larvae feed on floem and associated mycelia of ambrosia fungi and within one month (depending on the temperature conditions) reach their final size. Each larva then bores a pupation chamber and undergoes metamorphosis. Newly eclosed beetles take about two days to sclerotise before they emerge from the tree. Number of generations per year depends largely on temperature. While up to three generations are accomplished in lowland areas, the mountainous populations usually complete only one generation. Shortening of the day length (in the Central Europe to about 15 hrs in middle August (Schopf, 1985, 1989)) induces imaginal diapause. The development of immature stages is independent of the day length and is restricted only by lower temperature threshold of about 5°C (Annala, 1969). All eggs laid before the onset of diapause therefore yield larvae that complete their development into adults before the winter ensues. The adults usually feed before they retreat into the bark for hibernation. They emerge next spring when the temperature rises to about 7°C (Vité, 1952).

The present thesis attempts to fill some gaps in our knowledge of imaginal diapause in *Leptinotarsa decemlineata* and *Ips typographus*. Special attention is paid to the modifying role of temperature in both diapause induction and termination. The term “diapause intensity” is used as defined by Vinogradova (1974) and Košál (2006); it is shown that temperature during the pre-diapause and diapause stage strongly affects the spring emergence of the overwintering generation. Knowledge of this phenomenon is important for appropriate management of both studied pests.

REFERENCES

Annala E. (1969): Influence of temperature upon the development and voltinism of *Ips typographus* L. (Coleoptera, Scolytidae). Ann. Zool. Fenn. 6: 161 - 208.

- Constanzo J.P., Moore J.B., Lee R.E., Kaufman P.E., Wyman J.A.** (1997): Influence of soil hydric parameters on the winter cold hardiness of a burrowing beetle, *Leptinotarsa decemlineata* (Say), J. Comp. Physiol. 167: 169 – 176.
- Danks H.V.** (1987): Insect dormancy: An ecological perspective. Biological Survey of Canada (Terrestrial arthropods), Monograph, Series no. 1, 439 pp.
- de Kort, C.A.D.** (1990). 35 years of diapause research with the Colorado potato beetle. Entomol. Exp. Appl. 56: 1 - 13.
- de Wilde J.** (1955): The significance of the photoperiod for the occurrence of diapause in the adult *Leptinotarsa decemlineata* Say. Proc. First Int. Photobiol. Congr., pp. 96 – 101.
- de Wilde, J., Duintjer, S.C., Mook, L.** (1959). Physiology of diapause in the adult Colorado beetle (*Leptinotarsa decemlineata* Say) – I. The photoperiod as a controlling factor. J. Ins. Physiol. 3: 75 - 85.
- de Wilde, J., de Boehr, J.A.** (1961). Physiology of diapause in the adult Colorado beetle - II Diapause as a case of pseudo-allatectomy. J. Ins. Physiol. 6: 152 - 161.
- de Wilde J.** (1965): Photoperiod control of endocrines in insects. Arch. Anat. Microsc. Morphol. Exp. 54: 547 – 564.
- Denlinger D.L.** (1986): Dormancy in tropical insects. Ann. Rev. Entomol. 31: 239 – 264.
- Denlinger D.L.** (2002): Regulation of diapause. Ann. Rev. Entomol. 47: 93 – 122.
- Dortland J.F., Esch T.H.** (1970): A fine structural survey of the development of the adult fat body of *Leptinotarsa decemlineata*, J. Ins. Physiol. 16: (7) 1455 – 1466.
- Führer E.** (1981): Jahreszeitliche Qualitätsschwankungen des Fichtenbastes (*Picea excelsa* Link) als Brutsubstrat für den Borkenkäfer *Pityogenes chalcographus* L. (Coleoptera: Scolytidae). Z. angew. Entomol. 91: 74 - 83.
- Führer E., Chen Z.Y.** (1979): Zum Einfluss von Photoperiode und Temperatur auf die Entwicklung des Kupferstechers *Pityogenes chalcographus* L. Forstw. Cbl. 98: 87 - 91.
- Harper P.P., Hynes H.B.N.** (1970): Diapause in the nymphs of Canadian winter stoneflies. Ecology 51(4): 925 – 927.
- Košál V.** (2006): Eco-physiological phases of insect diapause. J. Ins. Physiol. 52: 113 – 127.
- Krausseopatz B., Kohler U., Schopf R.** (1995): The energetic state of *Ips typographus* (L.)(Col.; Scolytidae) during the life cycle. J. Appl. Ent. 119: 185 - 194.

- Lees A.D.** (1955): Physiology of diapause in Arthropods. Cambridge University Press, Cambridge. 151 pp.
- Mousseau T.A., Dingle H.** (1991): Maternal effects in insect life histories. *Ann. Rev. Entomol.* 36: 511 – 534.
- Musolin D.L., Saulich A.K.** (1996): Photoperiodic control of seasonal development in bugs (Heteroptera). *Entomol. Reviews* 76: 849 – 864.
- Numata H., Shiga S., Morita A.** (1997): Photoperiodic receptors in arthropods. *Zool. Sci.* 14: 187 – 197.
- Schneiderman H.A., Williams C.M.** (1953): The physiology of insect diapause. VII. The respiratory metabolism of the Cecropia silkworm during diapause and development. *Biol. Bull. Mar. Biol. Lab. Woods Hole* 105(2): 320 – 334.
- Schof A.** (1985): Zum Einfluss der Photoperiode auf die Entwicklung und Kältresistenz des Buchdruckers, *Ips typographus* (L.) (Coleoptera: Scolytidae). *Anz. Schädlingskde, Pflanzenschutz, Umweltschutz* 58: 73 - 75.
- Schof A.** (1989): Die Wirkung der Photoperiode auf die Induktion der Imaginaldiapause von *Ips typographus* (L.) (Coleoptera: Scolytidae). *J. Appl. Ent.* 107: 275 - 288.
- Tauber M.J., Tauber C.A.** (1976): Insect seasonality: diapause maintenance, termination, and postdiapause development. *Ann. Rev. Entomol.* 21: 81 – 107.
- Tauber M.J., Tauber C.A., Masaki S.** (1986): Seasonal adaptations of insects. Oxford University Press, Oxford. 411 pp.
- Tauber M.J., Tauber C.A.** (2002): Prolonged dormancy in *Leptinotarsa decemlineata* (Coleoptera : Chrysomelidae): A ten-year field study with implications for crop rotation. *Env. Entomol.* 31: 499 - 504.
- Ushatinskaya R.S.** (1981): Colorado potato beetle, *Leptinotarsa decemlineata* Say.: Phylogeny, Morphology, Physiology, Ecology, Adaptation, Natural enemies. USSR Academy of Sciences, Nauka Publishers, Moscow. 375 pp.
- Vinogradova E.B.** (1974): The pattern of reactivation of diapausing larvae in the blowfly, *Calliphora vicina*. *J. Ins. Physiol.* 20: 2487 – 2496.
- Vité J.P.** (1952): Temperaturversuche an *Ips typographus*. *Zool. Anz.* 149: 195 – 206.

- Wermelinger B., Seifert M.** (1998): Analysis of the temperature dependent development of the spruce bark beetle *Ips typographus* (L.) (Coleoptera: Scolytidae). J. Appl. Ent. 122: 185 - 191.
- Wermelinger B.** (2004): Ecology and management of the spruce bark beetle *Ips typographus* – a review of recent research. Forest Ecol. Managm. 202: 67 - 82.
- Wolda H., Denlinger D.L.** (1984): Diapause in a large aggregation of a tropical beetle. Econ. Ent. 9(2): 217 – 230.
- Zumr V.** (1985): Biologie a ekologie lýkožrouta smrkového (*Ips typographus*) a ochrana proti n ěmu – Academia; 124 p., Praha.
- Zumr V.** (1995): Lýkožrout smrkový – biologie, prevence a metody boje – Matice lesnická; 132 p., Praha.

CHAPTER 1

Doležal P., Sehmal F. (2007): Effects of photoperiod and temperature on the development and diapause of the bark beetle *Ips typographus*. J. Appl. Entomol. 131(3): 165 – 173.

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Abstract

Diapause was induced in a Central European population of *Ips typographus* grown at 20°C when the day length decreased below 16 h (50% diapause incidence occurred in the 14.7:9.3 h L:D (light:dark) regime). The non-diapausing adults fed on days 2-6 and 10-14 after the ecdysis and swarmed after the second feeding bout with chorionated eggs in the ovaries and sperm in the spermiducts. Neither gonads nor the flight muscles matured and no swarming occurred in the diapausing adults. The development from egg to adult took about 34 days in both 18:6 h (no diapause) and 12:12 h L:D (diapause) regimes but it was extended by up to 30% without diapause induction when only larvae or pupae were exposed to L:D 12:12 h. Diapause was induced in insects reared at L:D 12:12 h through the last larval and the pupal instars and/or in the adult stage. Temperature 23°C prevented diapause induction at L:D 12:12 h but diapause occurred at L:D 14:10 h associated with 26:6 °C thermoperiod. The effect of thermoperiods on the developmental rate requires further research. Exposure of the non-diapausing adults to 5°C for several days blocked feeding and evoked a diapause-like state, whereas diapausing adults fed and their gonads slowly developed at this temperature. Naturally diapausing adults exposed in forest to low night temperatures and transferred in October to 20°C readily reproduced at 18:6 but not 12:12 h L:D photoperiods. After 2-months at 5°C and darkness they became insensitive to the photoperiod, matured, and most of them also swarmed at 20°C in the 12:12 h L:D regime. In a Scandinavian population, diapause occurred at 18:6 h L:D and was terminated either by exposure to 5°C or by very long photoperiod (L:D 20:4 h) combined with high temperature (23°C).

Abstrakt

U st edoevropské populace lýkožrouta smrkového (*Ips typographus*) byla p i konstantní teplot 20° C diapauza indukována pokud délka dne poklesla pod 16 hodin (50% populace nastoupilo diapauzní vývoj p i délce dne 14,7 : 9,3 hodin (foto : skotofáze)). P íjem potravy byl u erstvých aktivních dosp lce rozd len do dvou cykl , z nichž první za al druhý den po imaginální ekdysi a trval do šestého dne, druhý pak probíhal od desátého do trnáctého dne. Po dokon ení druhého cyklu žíru vyletovali erství dosp lci, jejichž pohlavní orgány byly zcela zralé. Naproti tomu u diapauzních dosp lce nedošlo ke zrání pohlavních orgán ani rozvoji létacích sval a brouci neopoušt li vý ez, kde prod lali vývoj. Celková doba vývoje od vají ek do dosp lce ve fotoperiodách 18 : 6 hodin i 12 : 12 hodin trvala p ibližn 34 dní. K jejímu prodloužení až o 30% však došlo, pokud byly fotoperiod 12 : 12 hodin vystaveny larvy a kukly, p i emž diapauza nebyla indukována. K indukci diapauzy došlo pouze v p ípad , že fotoperiod 12 : 12 hodin byli lýkožrouti vystaveni trvale b hem stádií posledního larválního instaru, kukly a dosp lce, p ípadn pouze dosp lce. Pokud vývoj lýkožrout probíhal v teplot 23°C a vyšší, nedošlo k indukci diapauzy ani p i fotoperiod 12 : 12 hodin. Lýkožrouti chovaní v termoperiod 26° : 6° C a fotoperiod 14 : 10 hodin nastoupili diapauzní vývoj. Vliv r zných termoperiod na rychlost vývoje a indukci diapauzy vyžaduje další výzkum. Zatímco diapauzní dosp lci p íjmali potravu a jejich pohlavní orgány se postupn vyvíjely i v teplot 5°C, aktivní dosp lci vystavení t nto podmínkám p estali p íjímat potravu a po n kolika dnech se jejich chování za alo blížit diapauznímu. Diapauzní brouci, vystavení nízkým no ním teplotám a odebrání z p írody v polovin íjna, se rozmnožovali po p enesení do podmínek dlouhého dne 18 : 6 hodin, ale v podmínkách 12 : 12 hodin setrvali v diapauze. Teprve po dvou m sících v teplot 5°C ztratili brouci citlivost k fotoperiod a k rojení a rozmnožování došlo i v teplot 20°C a fotoperiod 12 : 12 hodin. Skandinávská populace lýkožrouta smrkového nastupovala diapauzní vývoj i v podmínkách 18 : 6 hodin. Experimenty prokázaly, že je možné tuto diapauzu ukon it bu p emíst ním do nízké teploty 5°C nebo velmi dlouhou fotoperiodou 20 : 4 hodin a vysokou teplotou 23°C.

Doležal P., Habuštová O., Selmal F. (2007): Effects of photoperiod and temperature on the rate of larval development, food conversion efficiency, and imaginal diapause in *Leptinotarsa decemlineata*.

Article accepted for publication in J. Ins. Physiol.

Podíl Petra Doležala na publikaci: 80%

Abstract

Larvae of *Leptinotarsa decemlineata* developed faster and consumed less food under short-day (Sd, 12:12 h light:darkness) than under long-day (Ld, 18:6 h L:D) conditions. The average index of food conversion efficiency was 5.4 in the Ld (25°C), and 7.2 and 11.9 (at 20°C and 25°C, respectively) in the Sd insects. Pupae were smaller under the Ld conditions due to a greater loss of biomass during the prepupal period that was nearly twice as long in the Sd insects. Virgin Ld females laid eggs for 6 months and survived 13 months. The lack of oviposition, reduced food intake, and behavioural changes characterised diapause in the Sd adults. Application of 100 µg JH III to newly eclosed adults was used to probe diapause intensity. At 25°C, the treatment elicited oviposition most effectively in females that were just transferred from the Ld to the Sd conditions. A distinctly lower response occurred in insects that had been kept under Sd conditions since hatching; their transfer to Ld conditions at the time of treatment had little effect on JH sensitivity. JH application to Sd females reared at 20°C caused enlargement of the germaria but no eggs were formed.

Abstrakt

Larvy mandelinky bramborové, *Leptinotarsa decemlineata* se vyvíjely rychleji a konzumovaly méně potravy v podmínkách krátkého dne (KD, 12 : 12 hodin, foto : skotofáze) než v podmínkách dlouhého dne (DD, 18 : 6 hodin, foto : skotofáze). U jedinců, kteří prodělali vývoj v podmínkách DD, byl index konverze živin průměrně 5,4, zatímco u jedinců z podmínek KD 7,2 (chov probíhal v teplotě 25°C) a 11,9 (chov probíhal v teplotě 20°C). Kukly z podmínek DD byly menší v důsledku zvýšených ztrát biomasy během stádia prepupy, které trvalo až dvakrát déle než v podmínkách KD. Neoplozené samice v podmínkách DD kladly vajíčka ještě šest měsíců po imaginální ekdysi a celkově se dožívaly stáří až 13 měsíců. Naproti tomu samice z podmínek KD nastoupily diapauzní vývoj, v jehož důsledkem byl omezený příjem potravy, nezralé pohlavní orgány a změny v chování. Intenzita diapauzy byla testována s pomocí aplikace 100 µg JH III na první dospělce z podmínek KD. Při teplotě 25°C měl JH III nejvyšší efekt (vyvolal ovipozici) na samice, které byly zároveň přemístěny z DD do podmínek KD. Mnohem méně se aplikace JH III projevila u jedinců, kteří prodělali celý vývoj v podmínkách KD. Aplikace JH III na samice z podmínek KD a teploty 20°C způsobila zvětšení germárií, ale nedošlo k formování vaječných komerek.

CHAPTER 2

Doležal P., Sehnal F. (2007): A simple method for the detection of imaginal diapause in beetles. *J. Appl. Entomol.* 131(3): 221 – 223.

Podíl Petra Doležala na publikaci: 80%

Abstract

Investigations on *Ips typographus* and *Leptinotarsa decemlineata* revealed that the activity of mitochondrial enzymes in the flight muscles provides a reliable criterion for the discrimination of diapausing and non-diapausing adults. Enzyme activity is conveniently detected *in situ* with commercially available tetrazolium salts. Staining intensity is correlated with the flight ability and often also with reproduction.

Abstrakt

Pokusy s lýkožroutem smrkovým, *Ips typographus*, a mandelinkou bramborovou, *Leptinotarsa decemlineata*, prokázaly, že aktivita mitochondriálních enzymů v létacích svalech může sloužit jako spolehlivý ukazatel k rozpoznání aktivních a diapauzních dospělých. Enzymatická aktivita je spolehlivě detekována *in situ* s pomocí komerčně dostupných tetrazoliových solí. Intenzita zbarvení létacích svalů je korelována se schopností letu a často rovněž s reprodukcí.

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CHAPTER 3

Proteolytic digestive enzymes of the larvae and adults of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) in relation to diapause

1. Introduction

The midgut pH values of most coleopteran larvae and adults are slightly acidic. This is consistent with low representation of the trypsin-like proteinases that dominate in the neutral or alkaline milieu of the digestive tract of most insects. A major part of the midgut proteolytic activity in beetles is due to the cystein-like endoproteinases (Murdock et al., 1987) and only a smaller portion is ascribed to the serin-like and aspartic proteinases (Wolfson and Murdock, 1990). The spectre of expressed midgut proteinases depends on the ingested food and may change in response to its quality and composition (Gruden et al., 2004). Such mechanism enables herbivorous pests to overcome the natural or artificially induced (e.g. by a genetic modification) plant defence based on the production of proteinase inhibitors (Confalonieri et al., 1998). Adults of the Colorado potato beetle express at least three types of proteinases that are resistant to all natural inhibitors present in the leaves of common potato cultivars (Gruden et al., 1998).

Most studies on protein digestion in *L. decemlineata* deal with the potency to overcome proteinase inhibition (Wolfson and Murdock, 1987; Gruden et al., 1998) or with the biochemical characteristics of proteinases (Thie and Houseman, 1990; Michaud et al., 1995). Nothing is known about processes that occur in the digestive tract during diapause and overwintering. Colorado potato beetles overwinter as diapausing adults and plasticity in the spectre of digestive proteinases prior to diapause is crucial for the accumulation of sufficient reserves that are essential for survival.

2. Material and methods

2.1. Insects

Adults of the Colorado potato beetles were collected in the vicinity of eské Bud jovice (Czech Republic, 49°N) in 2004. Standard culture was reared on Desirée potato cultivar under long-day conditions (18 : 6 hours, photophase : scotophase) and temperature 25°C.

Short-day conditions (12 : 12 hours, photophase : scotophase) and temperatures 20°C or 25°C were used for the induction of diapause. Last instar larvae or adults that had developed under the defined conditions (see above) since the egg were used in the experiment.

2.2. Sample preparation

Feeding larvae of the last instar or adults of various age were killed by decapitation and their midguts were dissected on ice. The midgut content, which was retained within the peritrophic membrane, was separated from the gut wall. Both parts were transferred to the eppendorf tubes that contained 50 µl of ice-cold Ringer solution. Each sample was mechanically homogenized by a glass pestle, sonicated for 30 seconds (BIOSONIC IIA, Bronwill Scientific, Rochester, NY, USA), and centrifuged at 18000 g for 10 minutes. The supernatants were collected and used immediately or frozen at -70°C.

2.3. Proteolytic activity assays

The total amount of proteins in each sample was estimated with BCA Protein Kit purchased from Sigma-Aldrich. Aliquots containing 25 µg protein were used for the assays.

General proteolytic activity was measured using azocasein resp. azocol as substrates. A 5 mg/ml azocasein solution was prepared in 50 mM Tris-HCl or 0.1 M HEPES-Imidazole buffers of desired pH. The reaction was started by mixing sample in 50 µl buffer with 200 µl azocasein solution and stopped after 90 min incubation at 37°C by adding 75 µl ice cold 20% TCA. The samples were mixed, kept in cold for 10 minutes, and centrifuged for 5 minutes at 18000 g. The supernatants were collected, mixed with 250 µl 1M NaOH, and transferred to a standard ELISA plate to read absorbance at 442 nm.

Azocoll assay was used for measuring general proteolytic activity at pH 4 to confirm the results of the azocasein assay. A suspension of 7.5 mg azocoll per ml (azocoll was dispersed in 50 mM Tris-HCl or 0.1 M HEPES-Imidazole, pH 4) was mixed for two hours, centrifuged, the supernatant discarded, and the sediment re-suspended to a final concentration 15 mg/ml. Subsequent steps, including preparation of control samples, were similar to the azocasein assay. The reaction time was 2 hours in 37°C and the absorbance was read at 490 nm.

Both the azocasein and the azocoll assays and both buffering systems proved suitable for the measurements. The use of Tris-HCl yielded slightly higher activity values than the HEPES buffer but the profile of activity at different pH was similar.

Two control samples were prepared at each pH tested – a blank (Tris, dd water, and substrate) and standard trypsin (Tris, trypsin 2.5 µg/ml, and substrate). Enzymatic activity of the sample was expressed as a percentage of trypsin activity after a correction for the blank samples. Specific substrates were used to analyse trypsin-like, chymotrypsin-like and aminopeptidase activities. To measure trypsin-like activity, 40 µl of the tissue extract was mixed with 160 µl N-Benzoyl-D,L-Arg-pNA (0,989 mM in 50 mM Tris-HCl or 0.1 M HEPES-Imidazole of defined pH) and incubated for 30 minutes at 30°C. The reaction was stopped by adding 50 µl 30% ice cold acetic acid and the substrate cleavage was measured on the ELISA plates as a change of absorbance at 405 nm. The chymotrypsin-like activity was tested in a similar way using 160 µl N-Suc-(Ala)₃-Pro-pNA solution (1 mM in 50 mM Tris-HCl or 0.1 M HEPES-Imidazole of defined pH) and 40 µl tissue extract. The aminopeptidase assay was also similar and included 160 µl L-Arg p-nitroanilid dihydrochlorid (15 mM solution in 50 mM Tris-HCl or 0.1 M HEPES-Imidazole of the defined pH) and 40 µl of the extract.

2.4. Inhibitory assays

Specific inhibitors were used to obtain additional information about the enzymatic activity. TLCK (0.5 mM in water) was used for the inhibition of cathepsins B and H and the trypsin-like proteinases, pepstatin (1 mM solution in methanol) for cathepsin D, E-64 (1 mM in water) for cathepsins B and H, and PMSF (100 mM in isopropanol) for the chymotrypsin-like enzymes. Midgut extracts (protein concentration 25 mg/ml) were pre-incubated with 5 µl of the respective inhibitor solution for 15 minutes prior to the proteinase activity assays. The decline of activity in comparison with samples without the inhibitors indicated which type of proteinases was involved.

3. Results

3.1. Control larvae, pupae and beetles from the long-day (18 : 6 hours, photo : scotophase, LD) conditions and temperature 25°C

In cultures kept under the LD conditions, adults started their maturation feeding within 24 hours after the imaginal ecdysis. At that time, the highest proteinase activity was recorded by azocol assay at pH 4. Activities at other pH values were negligible (**Fig 1**).

In the three days old and maximally feeding adults, the activity at pH 4 doubled and azocasein assay showed also an activity increase at pH 6 (considerably lower than the peak at pH 4) (**Fig 1**).

The highest increase of proteolytic activity was detected at pH 4 in the five days old adults, whereas proteolysis at pH 6 remained stable (**Fig 1**). No changes were recorded in the 7, 14 and 30 days old adults. The endoperitrophic samples always exhibited considerably higher activities than the midgut tissue samples. However, no difference in the spectre of proteinases was found between these two midgut compartments.

Specific inhibitor and substrate assays revealed that the peak of activity at pH 4 was reduced to about 50 % when the tissue extracts were pre-incubated with pepstatine. Similarly, activity at pH 6 decreased to less than 5 % after a simultaneous application of PMSF and E-64. E-64 proved to inhibit about 50 % of the activity, whereas TLCK only about 25 %.

The proteinase spectre in the midgut of larvae was not qualitatively different from that of the adults. The differences had only quantitative character. The peaks of activity were approximately 50 % lower in the midgut of the second and third instar larvae but similar as in adults in the fourth instar larvae. Midguts of prepupae and pupae showed only negligible activity at any pH.

3.2. Diapausing larvae, pupae and adults from the short-day (12 : 12 hours, photo : scotophase) photoperiod and temperatures 20° and 25° C

The profiles and activities of digestive proteinases in the midgut of the second, third and fourth instar larvae kept in the short-day cultures (SD, 12:12 h, light:dark) at 20° or 25°C were similar as in the corresponding stages of the LD culture. Proteolytic activity in the endoperitrophic compartment was higher than in the midgut wall at all rearing conditions and both in the larvae and adults. SD adults started feeding within 24 hours after the imaginal ecdysis at either 20° or 25°C. This was similar to the LD adults but the activity of digestive proteinases in the midgut of SD adults was lower. As shown for the 7- and 14-days

old adults (**Fig. 2**), the activity measured with either azocasein or the azocol assays at pH 4 decreased to approximately one third of the level detected in the LD adults. In contrary, azocasein assay detected an increase of activity at pH 6, so that proteinase activities at pH 4 and pH 6 were almost the same. Results of the inhibitor and specific substrate tests indicated that similar types of enzymes occurred in both LD and SD beetles.

None or negligible proteinase activity was detected in the midguts of sixty days old, non-feeding, diapausing beetles kept at 20°C or 25°C (**Fig. 3**). However, immediately after a resumption of their intermittent feeding, the proteolytic activity increased to a level similar to that in the 7- and 14-days old beetles.

Addition of midgut extracts from the non-feeding, diapausing SD adults suppressed proteolytic activity in the midgut extracts of the feeding, active adults from the LD conditions (**Fig. 4**).

4. Discussion

The results indicate that there are no qualitative differences in the spectre of proteinases between the active and diapausing larvae and adults of the Colorado potato beetle. Diapause intensity does not seem to influence the composition of digestive proteinases, although it strongly affects the behaviour and other physiological characteristics (Doležal et al., 2007). Four types of proteinase activities were biochemically detected in the midguts of active and diapausing larvae and adults. Proteinase(s) responsible for the notable peak of activity at pH 4 was strongly inhibited by pepstatin. Therefore we conclude, that the activity was largely due to an aspartic proteinase classified as cathepsin D. Consistent data were published by Thie and Houseman (1990), who further determined that this enzyme is active at pH 2.8 – 5, with an optimum at pH 4.5. Brunelle et al. (1999) found that this proteinase was responsible for the initial phase of the digestive processes. Bioassays with the larvae of the Colorado potato beetle proved that the inhibition of cathepsin D caused a slight decrease of the body weight and a remarkable prolongation of development in the third instar larvae (Brunelle et al., 2004).

Characterisation of the activity present at pH 6 was complicated by the fact that it was composed of three enzymes. Approximately 50% of the activity was inhibited by E-64, which is an effective inhibitor of cathepsins B and H. The remaining proteolytic activity was

inhibited by PMSF, which demonstrated presence of a chymotrypsin-type proteinase. Cysteine proteinases cathepsins B and H were described in the midgut of the Colorado potato beetle by Thie and Houseman (1990), who also characterised their optimal activity at pH 6 – 7.5. The detected chymotrypsin activity is probably due to the enzyme mentioned by Novillo et al. (1997). The enzyme was active at pH 5.5 – 6.5 and exhibited similarities with carboxypeptidase A.

Notable is the resumption of digestive enzyme activity in the diapausing beetles before the feeding bouts described by Doležal et al. (2007). Since midgut extracts from the non-feeding diapausing adults inhibit digestive enzymes in the extracts from the actively feeding adults, it is possible that midgut proteinases persist during diapause but their activities are curbed by endogenous proteinase inhibitors. Considering the role of cathepsin D in the initiative phases of digestion, the decrease of its activity after the induction of diapause may be one of the factors that suppress feeding in the diapausing beetles (Doležal et al., 2007).

Acknowledgements

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References

- Brunelle F., Nguyen-Quoc B., Cloutier C., Michaud D.** (1999): Protein Hydrolysis by Colorado Potato Beetle, *Leptinotarsa decemlineata*, Digestive Proteases: The Catalytic Role of Cathepsin D. Arch. Insect Biochem. Physiol. 42: 88 – 98.
- Brunelle F., Cloutier C., Michaud D.** (2004): Colorado Potato Beetles Compensate for Tomato Cathepsin D Inhibitor Expressed in Transgenic Potato. Arch. Insect Biochem. Physiol. 55: 103 – 113.
- Confalonieri M., Allegro G., Balestrazzi A., Fogher C., Dalledonne M.** (1998): Regeneration of *Populus nigra* Transgenic Plants Expressing a Kunitz Proteinase Inhibitor KTi3 Gene. Molecular Breeding 4(2): 137 – 145.

- Doležal P., Habuštová O., Sehnal F.** (2007): Photoperiod Affects the Rate of Development and Food Conversion Efficiency in the Larvae and Temperature Modulates Diapause Intensity in the Adults of *Leptinotarsa decemlineata*. J. Ins. Physiol. (in press).
- Gruden K., Štrukelj B., Popovi T., Lenar i B., Bevec T., Brzin J., Kregar I., Herzog-Velikonja J., Stiekema W.J., Bosch D., Jongsma M.A.** (1998): The Cysteine Protease Activity of Colorado Potato Beetle (*Leptinotarsa decemlineata* Say.) Guts, which is Insensitive to Potato Protease Inhibitors, is Inhibited by Thyroglobulin Type-1 Domain Inhibitors. Insect Biochem. Molec. Biol. 28: 549 – 560.
- Gruden K., Kuipers A.G.J., Gun ar G., Slapar N., Štrukelj B., Jongsma M.A.** (2004): Molecular Basis of Colorado Potato Beetle Adaptation to Potato Plant Defence at the Level of Digestive Cysteine Proteinases. Insect Biochem. Molec. Biol. 34: 365 – 375.
- Michaud D., Bernier-Vadnais N., Overney S., Yelle S.** (1995): Constitutive Expression of Digestive Cysteine Proteinase Forms During Development of the Colorado Potato Beetle, *Leptinotarsa decemlineata* Say. (Coleoptera; Chrysomelidae). Insect Biochem. Molec. Biol. 25(9): 1041 – 1048.
- Murdock L.L., Brookhart G., Dunn P.E., Foard D.E., Kelley S., Kitch L., Shade R.E., Shukle R.H., Wolfson J.L.** (1987): Cysteine Digestive Proteinases in Coleoptera. Comp. Biochem. Physiol. 87B: 783 – 787.
- Novillo C., Castanera P., Ortego F.** (1997): Characterization and Distribution of Chymotrypsin-Like and Other Digestive Proteases in Colorado Potato Beetle Larvae. Arch. Insect Biochem. Physiol. 36(3): 181 – 201.
- Thie N.M.R., Houseman J.G.** (1990): Identification of Cathepsin B, D and H in the Larval Midgut of Colorado Potato Beetle, *Leptinotarsa decemlineata* Say. (Coleoptera; Chrysomelidae). Insect Biochem. 20(3): 313 – 318.
- Wolfson J.L., Murdock L.L.** (1987): Suppression of Larval Colorado Potato Beetle Growth and Development by Digestive Proteinase Inhibitors. Entomol. Exp. Appl. 44: 235 – 240.
- Wolfson J.L., Murdock L.L.** (1990): Diversity in Digestive Proteinase Activity Among Insects. J. Chem. Ecol. 16: 1089 – 1102.

FIGURES

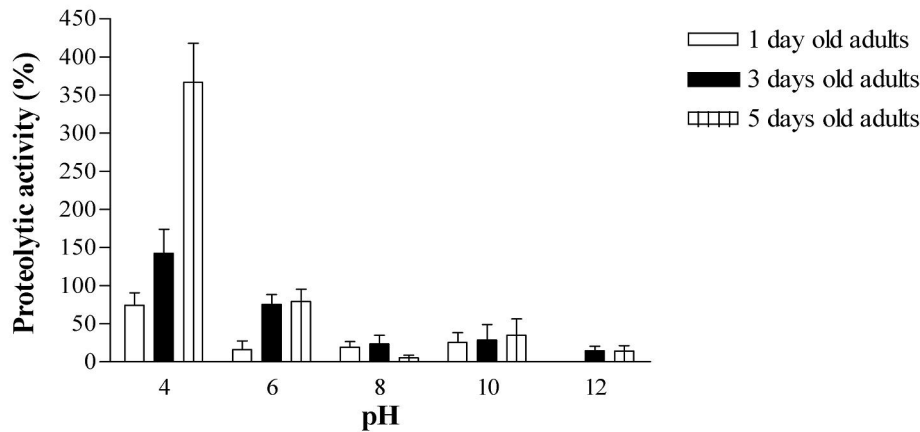


Fig. 1. Proteolytic activity, detected by the azocoll (pH 4) and azocasein (pH 6, 8, 10, 12) assays, in the midgut content of 1-, 3- and 5-day old active adults of *Leptinotarsa decemlineata*. The activity of samples was calculated as a percentage of the trypsin activity at defined pH. The thin lines show standard deviations. Notable is the increase at pH 4 and pH 6.

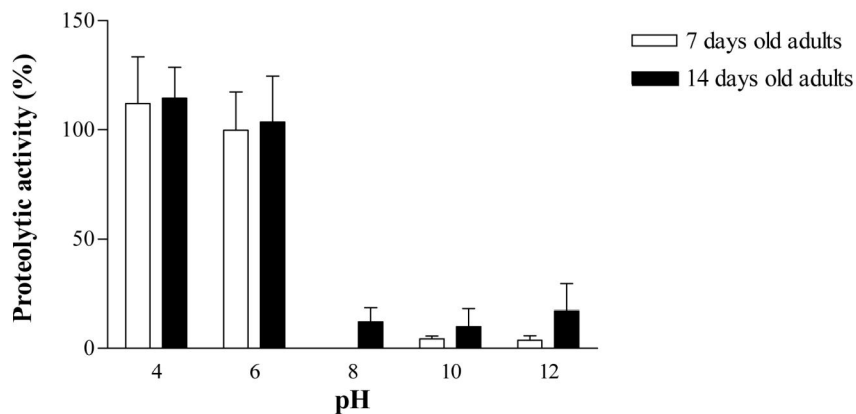


Fig. 2. Proteolytic activity, detected by the azocoll (pH 4) and azocasein (pH 6, 8, 10, 12) assays, in the midgut content of the 7- and 14-day old diapausing adults of *Leptinotarsa decemlineata*. The activity is expressed as a percentage of trypsin activity at defined pH. The thin lines show the standard deviations. Note the increase at pH 6 and the decrease at pH 4 in comparison with the active adults (**Fig 1**).

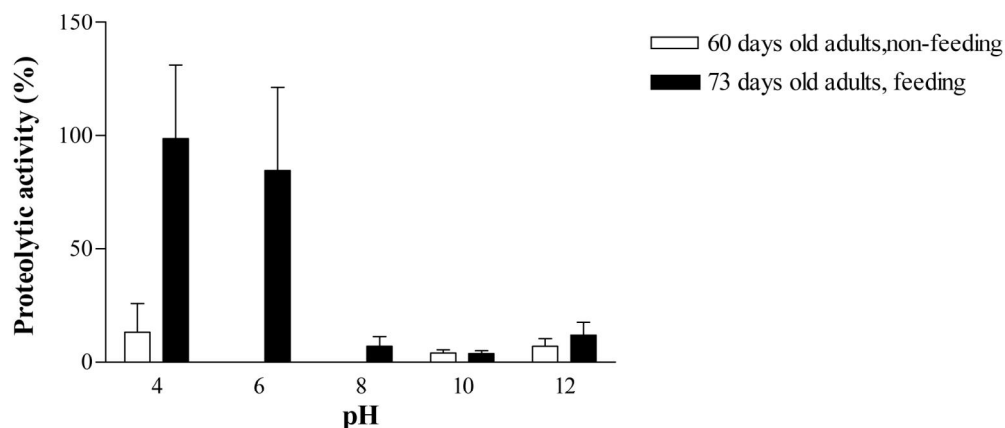


Fig. 3. Proteolytic activity, detected by the azocoll and azocasein assay, in the midgut content of the 60- and 73-day old diapausing adults of *Leptinotarsa decemlineata*. The activity is expressed as a percentage of trypsin activity at defined pH. The thin lines show the standard deviations. While the adults 60 days old did not feed and the proteolytic activity was very low, the 73 days old adults resumed feeding shortly before dissection and their proteolytic activity became comparable with that of the 7-day old diapausing adults (**Fig 2**).

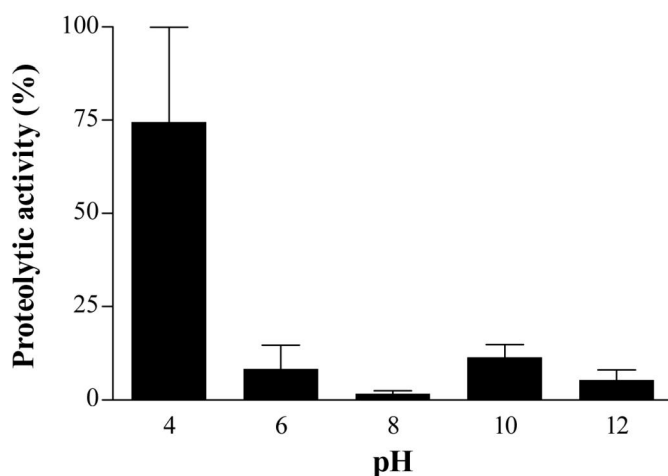


Fig. 4. A considerable decrease of proteolytic activity detected by the azocol and azocasein assay, when the midgut content samples of fully feeding active beetles were mixed with those of the non-feeding diapausing beetles. The thin lines show the standard deviations. Activity of samples was calculated as percentage of trypsin solution (2,5 µg/ml) activity at defined pH.

CONCLUSIONS

The thesis summarizes experimental data on the imaginal diapause of beetles *Ips typographus* (L.) (Curculionidae) and *Leptinotarsa decemlineata* (Say.) (Chrysomelidae). Modifying effect of temperature upon the entire diapause development is accentuated. A patented, simple method of diapause detection is described and research prospectives are briefly discussed. Here is a list of major findings:

- Critical day length inducing imaginal diapause in 50% individuals was assessed for the spruce bark beetle (*Ips typographus*) population from the Šumava National Park.
- Two cycles of feeding, separated by a starvation period, were observed in freshly ecdysed adults of the spruce bark beetle under laboratory conditions. During this time the beetles became sexually mature and ready to swarm. Since in nature the beetles may swarm already after the first feeding cycle (light coloured beetles), depending on the conditions of the bark, it is proposed that the interruption of feeding is an adaptation for dispersal.
- Sensitive period for the induction of diapause in the spruce bark beetles starts in the last larval instar. Exposure of the third instar larvae and pupae to the short-day conditions for an insufficient length of time does not induce diapause but causes a swarming delay of the non-diapausing beetles.
- High temperature prevented diapause induction in the spruce bark beetle despite the short-day conditions. The sensitivity to short photoperiod was retained in beetles exposed to temperature below developmental threshold during the scotophase.

- The food consumption rate of diapausing spruce bark beetles was affected by the photoperiod, demonstrating that the beetles percept changes in the day length.
- A distinct acceleration of larval, and especially prepupal development was induced by short photoperiod in the Colorado potato beetle, *Leptinotarsa decemlineata*.
- Body weight of adults that developed under short-day conditions was considerably higher then in those reared under the long-day photoperiod.
- Short-day conditions caused an increase of food conversion efficiency in the larvae of the Colorado potato beetle; this effect was augmented at reduced temperature.
- A strong effect of temperature upon diapause intensity was confirmed by the response of adults to topical application of JH III.
- Diapausing adults of both Colorado potato beetle and the spruce bark beetle occasionally feed when reared at temperatures higher than a threshold, demonstrating a remarkable plasticity of imaginal diapause.
- Diapausing and active adults can be distinguished from one another by the staining of flight muscles with tetrazolium salts.
- Digestive proteinases in the midgut of the active and diapausing adults and larvae of the Colorado potato beetle were briefly biochemically characterized and their activity tested.

RESEARCH PROSPECTIVES

Detail knowledge of all phases of the diapause development is crucial for understanding the bark beetle population dynamics and for planning adequate protective measures. Numerous attempts to predict spring emergence of the overwintering population of the spruce bark beetle have been made (Vité, 1952; Zumr, 1982; Coeln, 1996). The methods have largely been based on calculations of the thermal sums, which, however, do not reflect differences in the diapause intensity. Critical day length for the induction of diapause in the spruce bark beetle was assessed to be 14.7 : 9.3 hours (light : darkness) (Doležal and Sehnal, 2007b); it is likely that diapause intensity increases with day-length shortening below this threshold, as it occurs in course of the autumn months. Moreover, practically nothing is known about the role of temperature. Several methods were outlined to measure the intensity of diapause in adult insects. Doležal and Sehnal (2007a) proposed a method based on the measurement of cytochromoxidase activity in the flight muscles. This criterium is convenient for assessing diapause initiation and termination but it provides no information about changes in the course of diapause. The fat reserves content during oviposition, overwintering, spring emergence, and the maturation feeding was measured by Krausse-Opatz et al., (1995), who used it as a characteristic of general energetic state of the beetles. These authors also determined the levels of amino acids alanine and proline in the body of adult bark beetles. Proline is the most common source of energy for the flight muscles of beetles (Gäde and Auerswald, 1998). Since the increased titre of proline in active beetles is caused by the adipokinetic hormone (AKH), we assume that assessment of the AKH level could serve as an early indicator of diapause intensity. Measurements of the metabolic rate should also provide a useful criterion: lowered respiration during diapause was recorded in many insect species (Danks, 1987), including several weevils (Coleoptera; Curculionidae) (Brazzel and Newsom, 1959). Gehrken (1985) detected lower respiration rate in *Ips acuminatus* but did not evaluate possible correlation to the diapause intensity.

Previous research proved that diapausing beetles register changes in the environmental cues, e.g. temperature and day length, and are able to adjust their physiology and behavior in response to such changes. Two-months lasting exposure to 5°C and constant

darkness terminated imaginal diapause in most spruce bark beetles (Doležal and Sehnal, 2007b). The effect of low temperature upon the termination of diapause was documented in many insect species (Tauber et al., 1986; Hodek and Hodková, 1988; Košál, 2006). Similar data are available on the effect of temperature upon the induction and course of diapause. If we define diapause intensity as a relative developmental arrest (persistence of diapause) at given time under given environmental conditions (Vinogradova, 1974; Košál, 2006), it is obvious that the timing of spring emergence is strongly dependent on the diapause intensity and its changes during the harsh season. However, no general method for measuring the diapause intensity has been proposed so far.

Aims of future research:

- 1) Specify the relationship between diapause intensity and the daylength and temperature under laboratory conditions.
- 2) Define the criteria of assessing diapause intensity from the measurements of total lipid content, proteolytic enzyme activity in the midgut, total metabolic rate, and the titre of the adipokinetic hormone.
- 3) Check the influence of temperature range from -15°C to 20°C upon the behavior of diapausing and active adults (with the focus on feeding) and diapause termination. Examine if changes in winter temperature influence the developmental rate of spring bark beetle generation. Clarify the relationship between the age of adults and their response to various temperature regimes.
- 4) Follow the changes in diapause intensity in the field and correlate them with alterations in temperature and photoperiod during the pre-diapause, diapause and post-diapause.

References

- Brazzel J.R., Newsom L.D.** (1959): Diapause in *Anthonomus grandis* Boh. *J. Econ. Ent.* 52(4): 603 – 611.
- Coeln M., Yanzhang N., Führer E.** (1996): Entwicklung von Fichtenborkenkäfern in Abhängigkeit von thermischen Bedingungen verschiedener montaner Waldstufen (Coleoptera, Scolytidae). *Entomol. Gener.* 21(1/2): 37-54.

- Danks H.V.** (1987): Insect Dormancy: An Ecological Perspective. Biol. Survey Canada, Natl. Mus. Nat. Sci., Ottawa, 439 pp.
- Doležal P., Sehmal F.** (2007a): A simple method for the detection of imaginal diapause in beetles. J. Appl. Entomol. 131 (3): 221 - 223.
- Doležal P., Sehmal F.** (2007b): Effects of photoperiod and temperature on the development and diapause of the bark beetle *Ips typographus*. J. Appl. Entomol. 131 (3): 165 - 173.
- Gäde G., Auerswald L.** (1997): Insect neuropeptides regulating substrate mobilisation. S. Afr. J. Zool. 33: 65 – 70.
- Gehrken U.** (1985): Physiology of diapause in the adult bark beetle, *Ips acuminatus* Gyll., studied in relation to cold hardiness. J. Insect Physiol. 31: 909-916.
- Hodek I., Hodková M.** (1988): Multiple role of temperature during insect diapause: a review. Entomol. Exp. Appl. 49: 153 – 165.
- Košál V.** (2006): Eco-physiological phases of insect diapause. J. Insect Physiol. 52: 113 – 127.
- Krausse-Opatz B., Köhler U., Schopf R.** (1995): Zum energetischen Status von *Ips typographus* L. (Col., Scolytidae) während Jungkäferentwicklung, Überwinterung, Dispersion und Eiablage. J. Appl. Ent. 119: 185 – 194.
- Tauber M.J., Tauber C.A., Masaki S.** (1986): Seasonal adaptations of insects. Oxford Univ. Press, New York & Oxford, 411 pp.
- Vinogradova E.B.** (1974): The pattern of reactivation of diapausing larvae in the blowfly, *Calliphora vicina*. J. Ins. Physiol. 20: 2487 – 2496.
- Vité J. P.** (1952): Temperaturversuche an *Ips typographus* L. Zool. Anzeiger, 149: 195 – 206.
- Zumr V.** (1982): Podklady pro prognózu jarního rojení hlavních druhů k rovců (Coleoptera: Scolytidae) na smrku ztepilém (*Picea excelsa* L.). Lesnictví 28/11: 941-960.