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Ultrastructural and biochemical comparison of summer active and summer diapausing pupae of the horse chestnut leaf miner, *Cameraria ohridella* (Lepidoptera: Gracillariidae)

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Abstract. The ultrastructural and biochemical characteristics were studied in active and diapausing pupae of the horse chestnut leaf miner, *Cameraria ohridella*, collected in summer. The active pupae contained developing internal organs, while those in diapause mostly numerous non-differentiated cells packed with stored nutrients. These nutrients occurred in vacuoles as liquid material (lipids, proteins) or as reserve crystaloids (proteins). Triacylglycerols (TGs) were the main lipids stored by pupae and were more abundant in diapausing than active pupae. The amount of diacyglycerols (DGs) and phospholipids (PLs) was almost identical in both groups, which reflects the roles of these compounds in lipid transport and structure of cell membranes, respectively. A principal component analysis (PCA) indicated differences in the quality of the lipid compounds in both groups and that the TGs were mostly responsible for the difference. Polyunsaturated linolenic acid (18 : 3) was the most abundant fatty acid in both active and diapausing pupae, nevertheless its content was significantly higher in the former; the level of 7 fatty acids reached values higher than 1%. Differences in other nutrients were similar to those of TGs; higher contents of both glycogen and proteins were recorded in diapausing than active pupae. The results revealed significant differences between the two summer pupal forms of *C. ohridella* and furthered our understanding of the developmental strategy of this successful, invasive pest insect.

INTRODUCTION

The horse chestnut leaf miner, *Cameraria ohridella*, belongs to the lepidopteran family Gracillariidae. It feeds on horse chestnut, *Aesculus hyppocastanum*, where it lives inside leaves and feeds on their internal tissues; in temperate Europe it produces 3 or 4 generations per year (Skuhravý, 1998). Despite *C. ohridella* currently being one of the most important insect pest species of ornamental trees in Europe (Zemek et al., 2007a, b) the details of its bionomy and biology are poorly known.

The adults of *C. ohridella* are small brown moths with a white markings on their wings; they are about 4 mm long with a 7 mm wing span. The females lay eggs on the surface of leaves and after hatching the larvae burrow into to the assimilatory parenchyma of a leaf where they suck liquid from the cells and create typical cavities called mines (Weyda & Hrdý, 2002). The liquid excrements they deposit in the mines result in distinct circular spots about 1 cm in diameter. Photosynthesis of attacked leaves is reduced, which leads to a nutritional disturbance of the whole tree, defoliation, smaller seeds (Percival et al., 2011) and general weakening. At the end of their development the larvae of *C. ohridella* create pupal chambers (Skuhravý, 1998), each of which is enclosed in a solid parchment silk cocoon, which probably protects the insect against environmental changes, natural enemies and pathogens.

An interesting aspect of the development of *C. ohridella* is the occurrence of two types of pupae (Skuhravý, 1998; Samek, 2003; Hněvsová et al., 2011): (1) Active (non diapausing) pupae – typical of the generations that develop within the vegetative season. They are produced by the last instar larvae, which spin soft incomplete cocoons that consist of just a thin layer of silk containing substantially less material than the cocoons of the following group. These active pupae are not able to overwinter. (2) Diapausing pupae – their percentage is low in the first generation of the year, however, the percentage increases in the following generations (Samek, 2003). The larvae predestined to develop into diapausing pupae produce hard, complete cocoons that fully line the pupal chamber (Hněvsová et al., 2011). Only diapausing pupae are able to overwinter. The adults that emerge from these pupae eclose the following spring, the females lay eggs after mating and the developmental cycle starts again.

The phenomenon as to why diapausing pupae are formed in all generations within a year is not fully understood in terms of how it is controlled and the developmental or evolutional background is also unclear. Thus, the main aim of the present study was to

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describe certain morphological and biochemical differences between active and diapausing pupae and increase our understanding of the developmental strategy of *C. ohridella*.

MATERIAL AND METHODS

Cameraria ohridella pupae

Fresh horse chestnut leaves containing *C. ohridella* pupae were collected in the vicinity of České Budějovice (Czech Republic, 49°N) from 7th to 17th July 2012. The pupae were immediately dissected from the leaves and cocoons under a dissecting microscope and then checked to determine whether they were alive by stimulating them mechanically; dead pupae were discarded. Based on the type of cocoon and overall appearance (see below), the pupae were as either active or diapausing. Those used for the ultrastructural studies were processed immediately, while those used for the biochemical analyses were stored at –24°C until required. The diapausing pupae were homogenous, while the active pupae were more variable; individuals with pharate adult features were excluded from morphological studies, nevertheless, contamination of those used for biochemical analyses with these individuals cannot be excluded. However, this would have affected only the variability of the results, not their reliability.

Size of pupae

For measuring the size of the pupae a conventional flatbed scanner (Canon 8800F) was used; Petri dishes with pupae were inserted inside the scanner and scanned using direct optical (not interpolated) resolution (1200 dpi). The primary pictures were sharp enough to be suitable for the measuring their length. The length was measured on the pictures obtained using ruler-like software, which counts the number of pixels (Weyda, 2006).

Light and electron microscopy

For morphological and ultrastructural studies, optical and conventional transmission electron microscopy (TEM) were used. For this the freshly collected pupae were fixed in a mixture of 2% glutaraldehyde and 2.5% formaldehyde (EM grade) in 0.1M phosphate buffer, postfixed in 1% osmium tetroxide, dehydrated in a series of ethanols, embedded in epoxy resin (Spurr or mixture epon-araldite) and polymerized at 60°C. For optical microscopy semithin plastic sections (1 µm) were cut and stained with toluidine blue. For the TEM ultrathin sections were cut on Leitz ultramicrotome, stained with uranyl acetate-lead citrate and observed in Jeol 1010 transmission electron microscope.

Determination of lipids

Lipids were extracted from *C. ohridella* pupae using a chloroform : methanol (2 : 1) solution following the method of Folch et al. (1957) as modified by Košťál & Šimek (1998). Each pupa was homogenized in the solution and appropriate aliquots were used for the lipid determination. The procedure employed an ion trap LTQ mass spectrometer coupled to an Allegro ternary HPLC system equipped with an Accela autosampler with a thermostat chamber (all by Thermo, San Jose, CA, USA). The samples (5 µl) were separated using a Gemini column 250 × 2 mm i.d. 3 µm (Phenomenex, Torrance, CA, USA). The mobile phase consisted of (A) 5 mM ammonium acetate in methanol, (B) water and (C) 2-propanol. The analysis was completed within 80 minutes using a flow rate of 200 µl/min and the following gradient: 0–5 min 92% A and 8% B, 5–12 min 100% A, 12–50 min 100–40% A and 0–60% C, 50–65 min 40% A and 60% C, and 65–80 min back to the 92% A and 8% B. The column temperature was maintained at 30°C. The mass spectrometer was operated in the positive and negative ion detection modes at +4kV and –4kV with a capillary temperature of 220°C. The nitrogen was employed as a shield and auxiliary gas for both polarities. Mass range of 140–1400 Da was scanned every 0.5 s to obtain the full scan ESI mass spectra of lipids. For the investigation of the structures of the lipid molecules, the collisionally induced decomposition multi-stage ion trap tandem mass spectra MS^2 in both polarity settings were simultaneously recorded with a 3 Da isolation window. Maximum ion injection time was 100 ms and normalized collision energy 35%. The structure of each entity was identified using MS^2 experiments in positive or negative mode. Peak areas of detected lipids were used for estimating their relative content in the samples.

Fig. 1. The external appearance of the two types of pupae of *C. ohridella*. A – active pupa, B – diapausing pupa. Bar = 1 mm.

Determination of proteins

The proteins from the *C. ohridella* pupae were isolated using 0.2 M Tris-HCl buffer, pH 7.8, containing 1% triton. Each pupa was homogenized in this solution and the protein content measured in an appropriate aliquot using bicinchoninic acid reagent (Stoscheck, 1990). The bovine serum albumin standard curve was used to convert the optical densities of the samples measured at 562 nm into µg of protein.

Determination of glycogen

The glycogen content in the *C. ohridella* pupae was measured using the procedure of Ohtsu et al. (1992). The concentration of glucose liberated by the hydrolysis of glycogen was estimated using a modified anthrone method according to Carol et al. (1956) where the sample absorbance was read at 620 nm. A glucose standard curve was used to determine the quantity of glycogen.

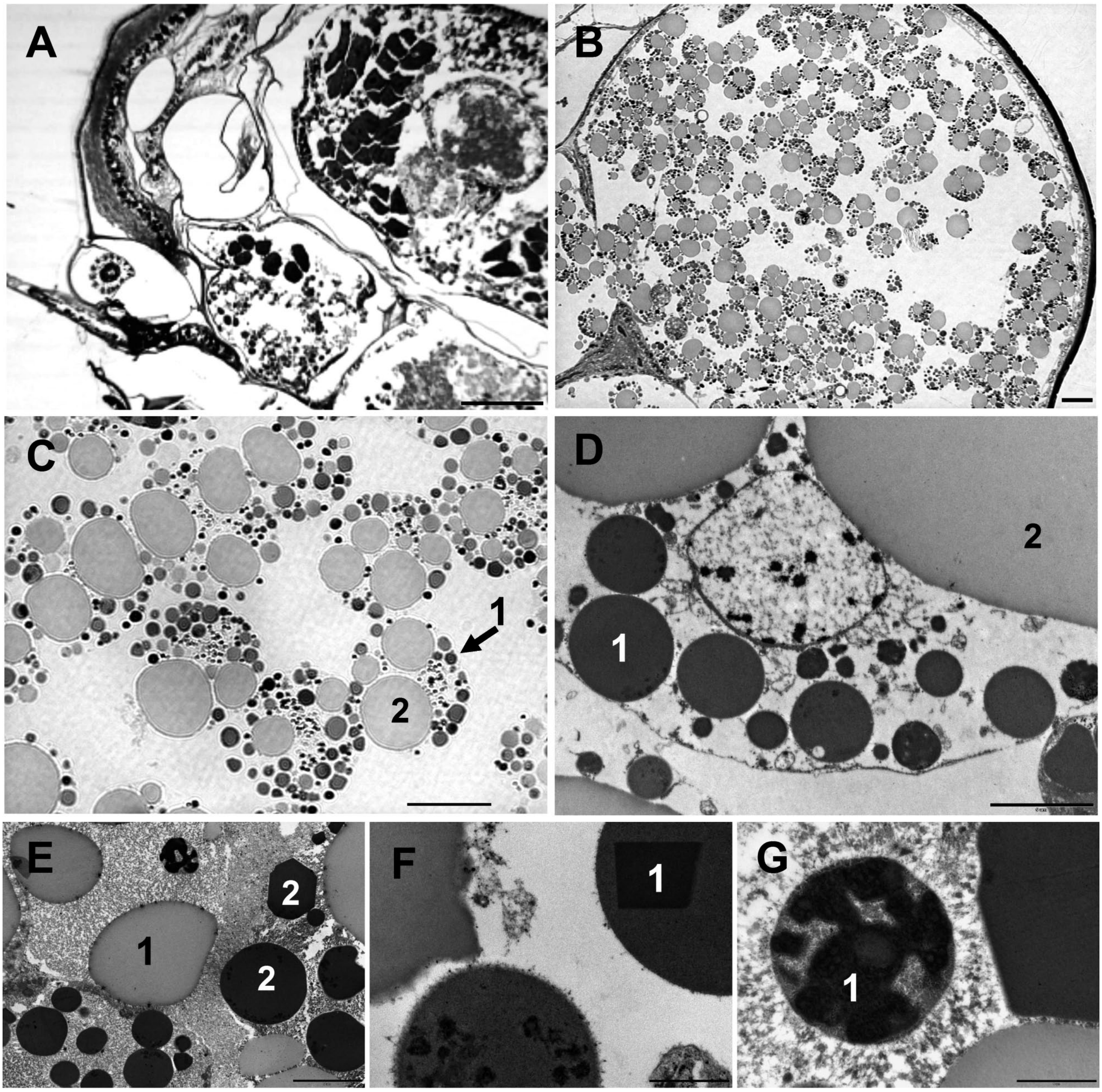

Fig. 2. Cross sections through pupae of *C. ohridella* showing the ultrastucture of storage cells. A, B, C – sections viewed using optical microscopy; D, E, F, G – sections viewed using transmission electron microscope (TEM). A – cross section of the body of an active pupa containing developing organs (half way through the development of an active pupa), bar = 100 µm; B – cross section of the body of a diapausing pupa containing storage cells (grey circles), bar = 100 µm; C – storage cells of a diapausing pupa containing vacuoles, bar = 20 µm, 1 – protein vacuole; 2 – lipid vacuole; D – details of a storage cell of a diapausing pupa, bar = 5 µm, 1 – protein vacuole; 2 – lipid vacuole; there is a nucleus cut tangentially in the centre of the photograph; E – ultrastructure of storage cells of a diapausing pupa, bar = 5 µm, 1 – lipid vacuole, 2 – several types of protein vacuoles; F – ultrastructure of storage cells of a diapausing pupa, bar = 2 µm, 1 – protein vacuole; G – heterogeneous protein vacuole (1) in a storage cell of a diapausing pupa, bar = 2 µm.

Data presentation and statistical analyses

The biochemical results were plotted using the graphic program Prism (GraphPad Software, version 6.01, San Diego, CA, USA). The bar graphs, Figs 3 (n = 5) and 5 (n = 6–11), present means ± SDs. For statistical evaluation of the results in Figs 3 and 5, and Table 1 see the corresponding legends.

Data obtained from the lipidomic HPLC/ESI/MS determination (active and diapausing pupae) were analyzed using Principal Component Analysis (PCA). For this analysis, the peak areas of compounds detected in total lipid extracts of *C. ohridella* pupae were used. Prior to the PCA analysis, the peak areas of 260 lipidic compounds were subjected to logarithmic transformation, scaling was focused on inter-species correlation, and the species scores were divided by their standard deviations and the data centred by species. In the PCA analyses, samples with similar chemical profiles were clustered together and segregated from those that were different. The statistical significance was assessed using redundancy analysis (RDA), a canonical variant of PCA, and the Monte Carlo permutation test (unrestricted permutations, n = 999); the identities of the tested groups (active and diapausing pupae)

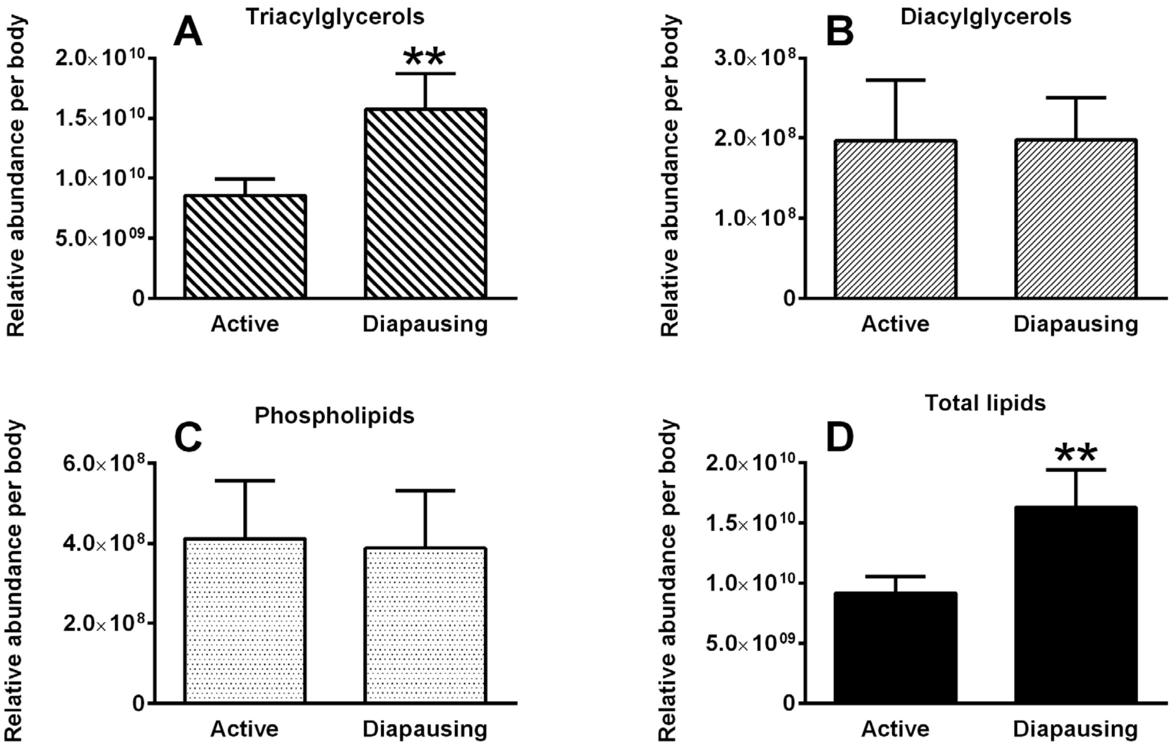

Fig. 3. Relative abundances of triacylglycerols (A), diacylglycerols (B), phospholipids (C) and total lipids (D) in the bodies of active and diapausing pupae of *C. ohridella*. Asterisks indicate significant differences between the active and diapausing pupae at $P < 0.01$ evaluated using Student's t-test; $n_1 = n_2 = 5$.

were used as a categorical predictor in the RDA analysis. This analysis also determined compounds responsible for the sample segregation. The multivariate data analysis software CANOCO 4.5 (Biometris, Plant Research International, Wageningen UR, The Netherlands) was used for both the PCA and RDA analyses.

RESULTS

Structural and ultrastructural characterization

This study revealed that the active and diapausing pupae of *C. ohridella* that appear simultaneously in summer differ in colour and size: the diapausing pupae were yellowish while the active pupae were brown (Fig. 1). In addition, the active pupae (3.46 ± 0.06 mm, n = 25) were significantly smaller (unpaired t-test, $p < 0.01$) than diapausing pupae (3.81 ± 0.02 mm, n = 25). In accordance with their size the active pupae (1.50 ± 0.28 mg, n = 10) were significantly lighter (unpaired t-test, $p < 0.01$) than diapausing pupae (1.93 ± 0.18 mg, n = 10).

In addition, substantial differences in organ and subcellular anatomy between both types of pupae were found using optical (histological semithin plastic sections of 1 µm thickness) and/or transmission electron microscopy. While active pupae contained developing internal organs (Fig. 2A) only numerous non-differentiated "storage" cells (Fig. 2B) were present in diapausing pupae. Nevertheless, these storage cells were rarely found in active pupae (data not shown). Typical storage cells were characterized by a circular shape and the presence of many vacuoles (Fig. 2C, D). Lighter and much bigger lipid vacuoles (their diameter was often more than 20 µm) were homogeneous, while darker protein vacuoles, mostly heterogenous, were much smaller with a diameter of 2–6 µm (Fig. 2C, D). Some of the vacuoles contained irregularly shaped condensed material and regularly shaped reserve crystaloids (Fig. 2E, F, G).

Nutritional reserves

The HPLC/MS analyses of the lipids extracted from *C. ohridella* pupae using chloroform-methanol generated a number of MS peaks, which indicated about 260 lipidic compounds (data not shown). For practical reasons, they were clustered into TGs, DGs and PLs (see also Material and Methods section). Their quantification revealed a significant difference between active and diapausing pupae in terms of TGs (Fig. 3A, P = 0.0031). Their level in diapausing pupae was about 1.8 higher than in active pupae. The lipids destined for direct utilization are ordinarily transported to the target tissues as DGs in insects; in accordance with this the DG content was similar in both types of pupae (Fig. 3B, P = 0.9796). A similar picture is shown in Fig. 3C, in which the level of PLs is depicted: no difference between the active and diapausing pupae was recorded (P = 0.8285). The significant difference in total lipids (data for TGs, DGs and PLs together – Fig. 3D, P = 0.0038) is not surprising as it reflects the difference in TGs and the levels of DGs and PLs were about 1–2 orders of magnitude less than that of TGs (Fig. 3), thus, their effect on the total lipid level was negligible. These findings were further confirmed by the PCA (Fig. 4). This statistical test indicated differences in the quality of particular lipidic compounds in the two groups and that TGs are mostly responsible for the difference. A RDA analysis confirmed the statistical significance of the difference at the $p = 0.018$ level (data not shown).

The above raises the question – Are the differences in the lipids in the two types of pupae also apparent at the level of fatty acids (FAs)? Indeed, the analysis indicated a significant selectivity in the accumulation and/or utilization of certain FAs by active and diapausing pupae (Table 1). Linolenic acid (18 : 3) was unequivocally the most abundant FA representing almost one half of all identified FAs. In addition, the following three FAs – oleic (18 : 1), linoleic (18 : 2) and palmitic (16 : 0), reached levels between 11–14%, respectively. The levels of another three FAs

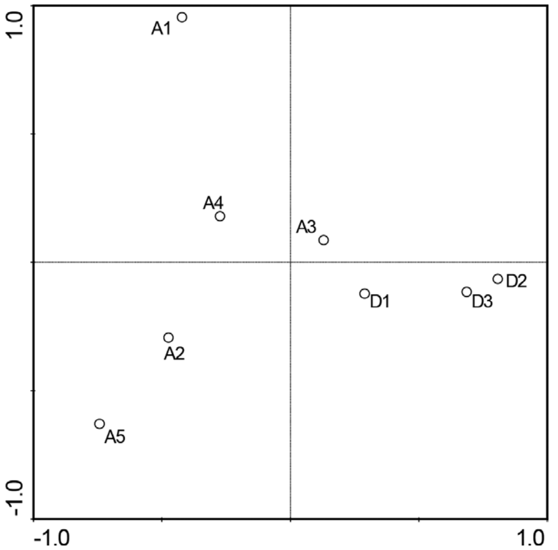

Fig. 4. The PCA evaluation of differences in lipid composition of active (A) and diapausing (D) pupae of *C. ohridella*. Subsequent RDA and Monte Carlo test (data not shown) indicated significance differences between these two types of pupae (A vs. D) at P = 0.018 level.

studied, stearic (18 : 0), palmitoleic (16 : 1) and margaric (17 : 0), were less than 10%, respectively; the FAs with levels of less than 1% were not considered. Further, there were significantly higher levels of palmitoleic (16 : 1) and linoleic (18 : 2) acids, and a lower level of linolenic (18 : 3) acid in diapausing than active pupae. The relatively lower level of linolenic acids (18 : 3) indicates that the role of these FAs might be more than only a source of energy during diapause.

Results of the analyses of the other nutrients in the two types of pupae studied were similar to those obtained for total lipid content, with 1.3 times more protein and 1.5 times more glycogen recorded in diapausing than active pupae (Fig. 5).

DISCUSSION

Within the last decade *C. ohridella* has gradually became a serious pest of horse chestnut trees in Europe, which has increased the interest in this small moth and revealed new facts about various aspects of its biology (Zemek et al., 2012; D'Costa et al., 2013; Irzykowska et al., 2013; Straw & Williams, 2013; Pocock & Evans, 2014). An interesting and important aspect of the life history strategy of *C. ohridella* is that it produces two different types of pupae throughout the vegetative period, active and diapausing (Skuhravý, 1998). It is surprising that the adaptive value of the parallel co-existence of diapausing and active pupae in summer is still unknown. Nevertheless, various interruptions in development (quiescence, diapause) in summer are well-known and well described in insects (Košťál, 2006). The summer diapause is a common phenomenon in insects: it occurs in all developmental stages and was recorded in all climatic regions. For example, within the Lepidoptera summer diapause is recorded in about 80 species, mostly occurring in pupal or adult stages (Masaki, 1980). There are several reasons for summer diapause, high temperature, drought, low food availability, predation pressure, synchronization and stabilization of development, nevertheless none of these account for diapausing and active pupae being produced at the same time by *C. ohridella*.

Table 1. Summary of the percentage (mean ± SD) of the most abundant fatty acids (FA) (reaching at least 1% content; their total content > 96%) in the total lipids (TGs, DGs, and PLs) in *C. ohridella* pupae. Statistically significant differences between the active (A) and diapausing (D) pupae for each FA were evaluated using Student's t-test. The arcsine square-root transformation (Sokal & Rohlf, 1969) was applied to normalize the percentage data before the statistical analysis. Significant differences (P < 0.05) are shown in bold.

Fatty acid	Pupae		Statistics		
	Active	Diapausing	*t*	df	*P*
16 : 0	12.89 ± 1.21	12.25 ± 1.03	0.7461	6	0.4838
16 : 1	**3.56 ± 1.08**	**5.40 ± 0.81**	2.5847	6	0.0415
17 : 0	0.96 ± 0.10	0.83 ± 0.10	1.6743	6	0.1451
18 : 0	8.40 ± 2.27	8.35 ± 1.78	0.0101	6	0.9922
18 : 1	11.66 ± 2.48	14.54 ± 1.87	1.7053	6	0.1390
18 : 2	**11.60 ± 0.88**	**13.59 ± 1.10**	2.8192	6	0.0304
18 : 3	**47.46 ± 3.45**	**41.34 ± 1.79**	2.7984	6	0.0312

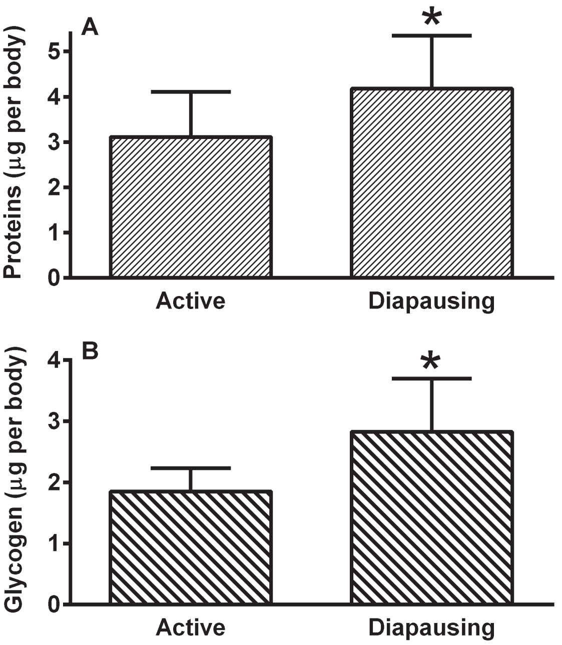

Fig. 5. Protein (A) and glycogen (B) levels recorded in the bodies of active and diapausing pupae of *C. ohridella*. Statistically significant differences at the 5% level between active and diapausing pupae evaluated using Student's t-test are indicated by an asterisk; n = 6–11.

Thus, it is apparent that a certain percentage of pupae in each generation of *C. ohridella* enter diapause although the environmental conditions are optimal for active life (Šefrová & Laštůvka, 2001). Only a small percentage (5–13%) enter diapause in the first spring generation (usually May), however, the percentage increases gradually in the following generations. In the second generation it reaches 20–35% and finally in the third and fourth generations, (usually in September or October, depending on the weather) most pupae diapause (Skuhravý, pers. commun., 2007). The percentage of pupae entering diapause is variable in each generation, which might be correlated with the availability of food as mentioned by Samek (2003), who also records that the percentage of pupae in diapause in the first generation is highly correlated with the population of this pest or the extent of leaf damage. Freise & Heitland (2002) even suggest that some pupae remain in diapause at least for three years.

The present study revealed that active and diapausing pupae also differed in their anatomy. The body cavity of both types of pupae contained approximately circular storage cells. They were very abundant in diapausing pupae and contained various nutrients mostly in vacuoles in the cells. It is believed that these nutrients support diapausing pupae during the whole non-feeding period, including overwintering. On the other hand, storage cells were rarely seen in active pupae and then mostly surrounding the rudiments of developing organs. It is probable that the nutrients are quickly utilized by actively developing pupae and that they provide the energy and structural components for metamorphosis, which ends about 10 days after pupation (Samek, 2003).

In addition, the active and diapausing pupae of *C. ohridella* differ also in the amount and quality of the silk in their cocoons (Hněvsová et al., 2011). The cocoons vary not only in macroscopic appearance and size, but in the quality of the silk. The major component of lepidopteran silk is fibroin, which is coated with several sericin proteins. This type of structure is typically associated with several small proteins with various functions. One, or several of the proteins work as protease inhibitors, which protect the cocoons against microbes (Nirmala et al., 2001a, b; Žurovec et al., 1998). Hněvsová et al. (2011) report that the inhibition of protease activity by an extract of the silk from active pupae is greater than by an extract of silk from diapausing pupae of *C. ohridella*. These authors suggest that the higher inhibitory activity in the silk of active pupae might reflect the necessity for a stronger protection of cocoon against microbes (and predatory insects) during the vegetative period than overwinter. In addition, pupae of *C. ohridella* have other sophisticated defence systems (Fiolka et al., 2005).

Successful overwintering requires the accumulation of a sufficient amount of energy rich nutrients within the vegetative season in order to survive the following non-feeding period. Accordingly, the total lipid level in diapausing pupae was significantly greater than in active pupae. Triacylglycerols (TGs), which are an important reserve of energy in insects [in diapausing insects they make up as much as 80–95% of total lipid content (Hahn & Denlinger, 2011)], were also the dominant lipids in *C. ohridella*. The contents of the other classes of lipids were much lower (see Fig. 3). It is not surprising that the level of diacylglycerols (DGs) was almost identical in active and diapausing pupae. The DGs serve mainly as transport lipids in insects (Van der Horst, 1982; Arrese et al., 1996) and their level is more or less independent of the total lipid level. A similar situation was recorded for phospholipids (PLs). This interesting class of lipids is an important structural component of cell membranes and affects their characteristics, which is important for the survival of insects at low temperatures. Both of the types of pupae studied were collected in the field at the same time of year and had both experienced identical temperature conditions, which probably accounts for the same level of PLs in both active and diapausing pupae. Whether the level of PLs in diapausing pupae of *C. ohridella* changes during autumn and winter, when exterior temperature can be very low, is unknown and remains to be elucidated.

Fatty acids (FAs) are the main components of lipids. Several dozen FAs are described in insects, however, 8–9 FAs with 12–18 carbon atoms in the molecule make up the major proportion. The saturated FAs are lauric (12 : 0), myristic (14 : 0), palmitic (16 : 0) and stearic (18 : 0), the monounsaturated FAs myristoleic (14 : 1), palmitoleic (16 : 1) and oleic (18 : 1) and polyunsaturated FAs linoleic (18 : 2) and linolenic (18 : 3) (Downer, 1985; Schneider & Dorn, 1994). The most common FAs in *C. ohridella* are included in these categories (see Table 1). Linolenic acid seems to be the most abundant in both pupal groups and is significantly more abundant in active than diapausing pupae, which indicates it is not only a source of energy. A high level of linolenic acid is also recorded in the locust, *Locusta migratoria* (Tomčala et al., 2010) and firebug, *Pyrrhocoris apterus* (Bártů et al., 2010). Oleic and linoleic acids, however, are the most abundant FAs in the latter two species, respectively. Level of the next two FAs in *C. ohridella*, palmitoleic and linoleic, is slightly, but significantly higher in diapausing pupae, which might indicate they are more important energy reserves for the long non-feeding period.

The determination of the levels of the other nutrients in *C. ohridella*, glycogen and proteins, did not result in any surprizes. As in the case of lipids their level was higher in diapausing pupae. Fat body glycogen generally has two main roles in insect diapause: (1) an energy reserve supplementing the role of TGs, and (2) a source of various cryoprotectants (Hahn & Denlinger, 2011). Whether the latter role is important for pupae of *C. ohridella* is unknown. Diapausing insects also accumulate proteins (mostly in the form of hexamerins), which probably have a role in anabolic activities and turnover of amino acids, some of which may play a role in nutrient storage (Hahn & Denlinger, 2011). Again, the details of the role of proteins in diapausing pupae of *C. ohridella* are unknown.

The morphometric evaluation of the pupae revealed that the diapausing pupae were significantly larger and heavier than non diapausing pupae. It is likely that the maximum levels of nutrients (lipids, glycogen, proteins) in pupae are limited and diapausing pupae are not able to increase their concentrations substantially (data not shown). To overcome this limitation diapausing pupae are larger and heavier and so accumulate more nutrients for a much longer non-feeding period than experienced by active pupae.

In summary, this paper deals with structural and biochemical characteristics of *C. ohridella* pupae. This study revealed significant differences in anatomy, cell structure and ultrastructure, and in the quantity and quality of nutrients in the active and diapausing pupae that develop in summer. However, the adaptive significance of producing two types of pupae in summer is unknown. It apparently represents one of evolutionary adaptations making this species so successful.

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